

Credible politics versus incredible consensus

Open and even scientifically contentious debates about policy issues are, on balance, beneficial. In that context, recent signals from Europe are encouraging, whereas those from the US Congress are dispiriting.

Scientific research is a human pursuit in which both passionate commitment to conflicting ideas and personal antipathies can lead to friction between those pursuing the same goal. Most would agree that an objective reality will eventually be reached, and that ultimately it is this that counts. But such arguments, especially when based on genuine intellectual differences, loom large when the scientific understanding critical to political and regulatory decisions is incomplete. If the public handling of such controversies is an indicator of political maturity, one country at least is showing signs of regression.

Scientific protagonists in policy debates who have a real or perceived non-scientific agenda do not help. For that reason, moves to circumvent such problems by Jacques Santer, president of the European Commission (EC), are welcome, if belated. His wish to distance the EC's suppliers of scientific advice relating to health and consumer protection from directorates associated with supply industries may boost the public's shaky confidence in Brussels procedures (see page 285). His bid for more openness in their proceedings could also help to avoid situations in which conflicting interests lead to bad practice.

The perception of a non-scientific agenda has also undermined the ability of Nirex, the UK agency responsible for the long-term disposal of nuclear waste, to make its case for the development of an underground repository. Last week's report by the Radioactive Waste Management Advisory Committee on Nirex's peer-review procedures highlighted the boost of confidence in that organization's scientific thinking on issues such as rock hydrology that followed an independent review by a panel appointed by the Royal Society. It is, in fact, not easy to find scientists with relevant expertise who have not at some point been associated either with Nirex or its environmentalist opponents. But a learned society is in as good a position as anyone to stand back and deliver an impartial assessment

ment incorporating what they have to say.

Involving academies and learned societies in order to increase public confidence has its own difficulties, however. Their members will not (and should not) take kindly to the adoption of any recommendation that goes beyond what is scientifically uncontroversial. And a new potential obstacle has emerged in the United States, following a legal ruling that, where its advice has been sought by government agencies, the National Academy of Sciences must conduct its deliberations in public. The academy is likely to appeal, stating that this would tie its hands by forcing scientists to make a public defence of views which they may prefer to express privately.

Even with the most perfectly balanced and disinterested statement of current knowledge and uncertainties, there is another step to be taken before reaching any political or regulatory decision. Where the costs are obvious and the evidence of benefits is ambiguous, contention frequently arises. The stage is set for just such a controversy in a forthcoming debate in the US Congress about proposed regulations to decrease ground-level concentrations of ozone and particles in the atmosphere. A surprising weakness in the political process is emerging. Members of Congress, as their staff members admit (see page 284), often fail to distinguish between scientific uncertainties and political controversies. For them, open debate of scientific differences is seen as damaging to a belief that science can contribute usefully. Admittedly such debates can be hijacked by pressure groups. But they also have the potential virtues of increasing public understanding of and confidence in the eventual decisions.

Just as overly secretive and interest-bound bodies in Europe are becoming more open and independent, the country that many Europeans see as a model of open debate seems to be becoming increasingly reluctant to deal with intellectual contention in a mature manner. □

Xenotransplantation: the heart of the matter

The BSE crisis has concentrated the British government's thinking about xenotransplantation.

What a difference a year makes. It is just over twelve months since the British government rejected a proposal from a parliamentary committee to set up a statutory body to regulate genetic research and its applications. Now, after the crisis over bovine spongiform encephalopathy (BSE), it is singing a different tune, promising tough regulations to protect individuals from the potential dangers of transplanting organs from animals to humans. Last week's announcement of a Xenotransplantation Interim Regulatory Authority reflects the thoroughness with which an advisory group set up to examine the ethics involved has done its task (see page 285). For it needs to be acknowledged more widely that there are public, and not just personal, health issues at stake.

Much current concern focuses on the possible transmission of dormant retroviruses from animals to man, perhaps to become expressed only after several years. If this were to happen, there is a theoretical

chance that the virus could spread to other individuals from those receiving transplants. The AIDS epidemic, which is generally believed to have originated in a monkey virus, is a gruesome reminder of the potential consequences.

Caution is therefore to be applauded. A key issue to be faced by the new regulatory body is whether the apparent lack of expression of retroviruses in monkeys used in experimental transplantation of pig organs is sufficient to allow clinical trials on humans, or whether these should be delayed until we have sufficient detailed knowledge of the pig genome to 'breed out' the offending DNA. While commercial (and even medical) pressure may favour the first, there are good reasons for adopting the second. The same philosophy could beneficially be followed by other agencies, such as the US Food and Drug Administration, which have been considering more moderate measures. The one lesson to be learnt from the recent BSE crisis is "never say 'never'". □



Third world agricultural centres face a wave of redundancies

[NEW DELHI & LONDON] Growing competitive pressures on internationally funded agricultural research in the developing world has led to severe staff reductions at major research centres in India and the Philippines, with the prospect of layoffs at a third centre in Colombia.

Last week, the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), near Hyderabad in India, confirmed that 19 of its 81 research staff have been made redundant. And at least another 500 — one-third of the nationally hired staff — are expected to be served notice in the next few weeks.

The move follows an announcement earlier this month that the International Rice Research Institute (IRRI), at Los Baños in the Philippines, was issuing 'separation' notices to 550 individuals — half of its core and mainly local staff. IRRI suffered a deficit of US\$2 million last year and faces a projected cut of \$6 million in its budget for 1997. Somewhat controversially, no non-Filipino has been asked to leave.

It is widely believed that the Centro Internacional de Agricultura Tropical (CIAT), at Cali in Colombia, is next in line for staff cuts.

The three centres are among 16 institutions supported by the 26-year-old Consul-

tative Group on International Agricultural Research (CGIAR), a consortium of public and private donor agencies organized through the World Bank. Most of the centres are based in the developing world.

IRRI is the flagship institute of the consortium, with the biggest budget (\$40.2 million in 1995). It carries out research on rice production worldwide. ICRISAT, the third-largest institute in the CGIAR system, has been trying to improve crops such as sorghum and millets that grow in arid zones.

James G. Ryan, director general of ICRISAT, said of his institute's staff reductions last week that "we regret [having] to do this but it has become inevitable". He says the institute, whose annual running costs are about \$33 million, faces a shortfall of \$4 million in 1997 following a major cut in funding from donor countries. The gap is likely to grow to \$8 million next year if remedial action is not taken.

The institute has already cut some research programmes in Africa and Latin America. It also introduced a voluntary redundancy scheme in 1994, which helped to cut the workforce by a third, down from a peak of 2,895 in 1991 to 1,737 in 1996.

The centres' troubles can be traced to 1992–93, when a cut of \$8 million in US

Ranking of contributions to research at CGIAR centres (\$ million)

Donor	1991	1995
US	45.63	32.08 (3)
World Bank	35.11	50.02 (1)
Japan	23.70	33.94 (2)
Canada	15.73	12.70 (7)
EC	13.45	16.73 (4)
UK	11.57	9.86 (10)
Germany	11.04	15.84 (5)
Switzerland	10.16	11.94 (8)
UNDP	6.64	8.42 (11)
Netherlands	6.45	12.82 (6)
Denmark	3.39 (17)	10.02 (9)

donations triggered a fall totalling \$13 million in donor support for CGIAR research. As a result, income for the 16 centres dropped from \$247 million in 1992 to \$234 million in 1993.

The World Bank agreed to inject \$20 million in emergency assistance. And an extra \$12.6 million from other countries helped to restore a degree of balance the following year. But the United States continued to make deep cuts in its annual donation, which had fallen to \$26 million at the end of 1996, compared to \$48 million four years earlier.

By 1994, faced with potential collapse, the management of CGIAR launched an 18-month review of its research agenda and funding arrangements. Funding increased to \$302 million in 1996, largely thanks to the World Bank, Japan and the European Union (see table above).

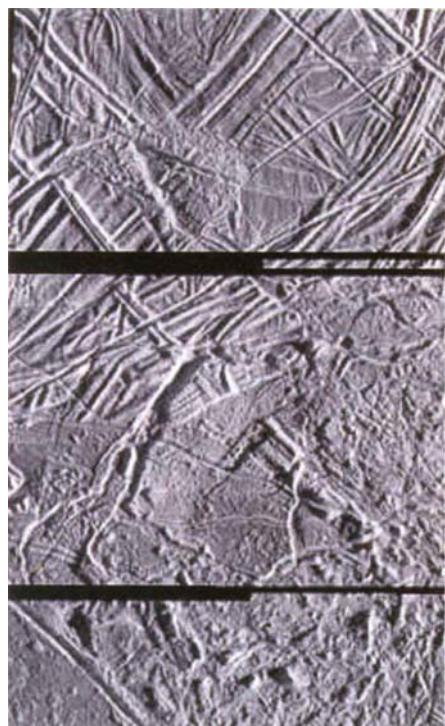
The 1997 allocation is \$340 million. But research income is no longer guaranteed, and centres now have to compete for money. They are required to investigate alternative funding sources and to pursue more collaborative ventures with other centres and with national agricultural research councils.

The changes proved controversial. The International Maize and Wheat Improvement Center (CIMMYT) in Mexico, was among the first institutions to be affected. Research income has fallen from \$27.7 million in 1991 to \$22.3 million in 1995. Research staff have been cut from 85 to 65.

Walter Falcon, chairman of CIMMYT, says funds are "tight". But he says that the worst times have passed, and that the changes — which he describes as "a second-best solution" — have been painful, and could lead to a reduction in research.

He says many centres are not used to fund-raising, and research time and budgets are being further constrained by the need for collaboration between distant centres. But Falcon points out that the centres could not

Icy complexion of Jupiter's frozen moon



[WASHINGTON] The relative lack of impact craters on the surface of Jupiter's frozen moon Europa indicates that much of the surface is relatively young, according to researchers at the National Aeronautics and Space Administration's Jet Propulsion Laboratory in Pasadena, California, who last week released the first close-up pictures taken by the Galileo spacecraft.

A composite picture of a swathe 11 km wide, taken last December during the spacecraft's fourth orbit of the planet (left), shows the surface of Europa to be structurally complex, with ice volcanoes and signs of ice flows that probably originated from them, as well as evidence of the movement of tectonic plates.

According to Ronald Greeley of Arizona State University, the images appear to increase the likelihood that Europa is one of the few locations in the Solar System that could have hosted the development of life. But the pictures so far produced by Galileo have not allowed scientists to answer one of the key questions they face — whether a subterranean ocean still exists. □

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Testing times: 550 staff at IRRI, the rice research centre, are receiving 'separation' notices.

remain immune from 'donor fatigue' and the introduction of competition in the funding of research, particularly in the developed world. Ryan concurs by saying that a smaller workforce will lead to ICRISAT's research activities being sacrificed and could, if financial problems persist, lead to closure of some of its units in Africa.

But one source close to the agricultural research centre system believes that the cutbacks at IRRI will be less harmful, as they have been designed partly to jolt Asian countries into contributing more to the centre given that "95 per cent of the world's rice is grown in Asia". He says the cutbacks will not affect research at IRRI as much as anticipated. "IRRI was overstaffed, and a lot of pruning was needed."

Employees at centres hit by the cuts are concerned about the size of the separation pay package. Ryan says that this will be announced after it has been approved by ICRISAT's governing board, which is scheduled to meet at the end of February.

ICRISAT's local Indian staff feel that a confrontation with management over a separation package may be unavoidable because of the institute's refusal to negotiate with its employees about an agreed formula for severance terms.

Local staff, most of whom have 15 to 25 years service, are demanding a minimum of three months' salary for each year of service. Axed Filipino staff at IRRI are to receive between one and two months' salary for each year of service, plus other entitlements.

R. S. Paroda, director general of the Indian Council of Agricultural Research and a member of the ICRISAT board, declined to comment on the sackings as this might be seen as interfering with the institute's autonomy. But he said that his council may employ former ICRISAT staff. Both Paroda and Ryan ruled out speculation that a lack of support could signal the end of ICRISAT's operations in India. "I do not think the fund cuts were directed against ICRISAT," says Ryan, adding that several other CGIAR centres face a similar situation. **K. S. Jayaraman & Ehsan Masood**

Nuclear waste peer review 'needs more transparency'

[LONDON] Scientists advising the UK government on radioactive waste disposal have called on the waste management agency Nirex to open up its peer-review arrangements to independent scrutiny if it wants to retain public confidence in its activities.

In a report assessing Nirex's proposals to publish and peer review its science, members of the government's Radioactive Waste Management Advisory Committee (RWMAC) endorse the efforts of Nirex scientists to achieve greater transparency. But they say the peer-review arrangements need revising.

The report appears at a critical time for Nirex. The waste agency is engaged in a heated dispute about the integrity of its public information following the leak of an internal memorandum last week which raises questions about the adequacy of data about the site at Sellafield, in the northwest of England, where Nirex plans to bury waste from nuclear power stations.

Nirex's scientific work is currently assessed by a review panel of external scientists appointed and paid for by the agency. But the RWMAC report, which was published last Tuesday (21 January), says there still remains a perception that the review panel is not fully independent from Nirex. The committee voiced "a residual concern" about the effect this might have on public confidence in the disposal of nuclear waste.

The report suggests that Nirex publish a twice-yearly review report in addition to its proposed biennial document. The report also suggests that learned bodies, such as the Royal Society of Chemistry, should periodically review Nirex's science, and that the agency should submit more of its research to peer-reviewed journals.

Stephen Sparks, a professor of geology at the University of Bristol and former member of Nirex's review panel, says the panel is not afraid to be critical when offering advice. But he acknowledges that the panel's findings are published as "broad suggestions", omitting "the nitty-gritty details".

Sparks says he welcomes the idea of independent assessment of Nirex research. The authority of independent assessors will exceed that of paid consultants, he says, "even if it is the same people". John Holmes, Nirex's director for science, also says he is "content with the report's conclusions".

The memorandum leaked last week, of which Holmes was the author, appears to conflict with the agency's earlier statements. For example, Nirex has repeatedly rejected claims from other scientists and environmentalist groups that more data about the geology and hydrogeology of the Sellafield

site are needed before it proceeds with plans to build an experimental underground rock laboratory needed for research into the proposed site (see *Nature* 383, 751; 1996).

Yet critics say that uncertainty still remains about the movement of radioactive leaks from buried waste through the underground rock, and the possibility that this radioactivity may return to the surface.

In the memorandum, which is dated 10 December 1996, Holmes says he is "concerned" that having spent £200 million (US\$320 million), "the modellers are saying that we are short of datapoints by a factor of 10x or 100x". He concludes by suggesting "more site characterization" as one of three options "to get to the bottom of this".

Both statements appear to conflict with evidence given to a public inquiry into the rock laboratory last year at which Holmes said that "it is considered that remaining surface-based activities, while necessary to complete our picture, are very unlikely to substantially change our view of the site".

Holmes says that his more recent memorandum refers to "off-the-cuff comments" made by a modeller and does not acknowledge the need to postpone the rock laboratory and obtain more data instead by drilling additional boreholes. On the contrary, he says, it refers to "the law of diminishing returns" regarding the usefulness of data from boreholes, and that the option of "more site characterization" refers to the need to build the rock laboratory.

Further controversy has been generated by a reference in the memorandum to the contentious issue of the permeability of the site rock to ground water. Holmes writes: "We need to look hard at the [rock] permeability to see if we can justify a lower central [average] value (if not, I have the feeling that we may struggle to make a case for the site)."

Rachel Western, nuclear researcher at Friends of the Earth, argues that this statement suggests that Nirex is "cooking the books". Even Sir John Knill, a former chairman of RWMAC, says that Nirex appears to be "suggesting the goalposts could be moved". According to Knill, the central permeability value is less of an issue, as most groundwater flow will take place at points of high permeability in the rock. He says that 50 per cent of Nirex's permeability values are higher than the central value, and 10 per cent are 100 times higher.

But Holmes says the statement in the memorandum refers to a previous model of rock permeability in which modellers had used an average value of permeability considered by Nirex to be high. **Ehsan Masood**

'Real' fall in US research funding revealed

[WASHINGTON] US spending on research and development (R&D) has fallen by 5 per cent since 1994, considerably more than the 2 per cent drop acknowledged by the Clinton administration, according to the National Academy of Sciences.

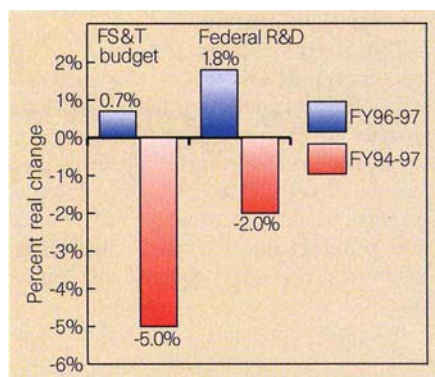
But the shrinkage has been concealed in official statistics which include non-research items — such as the testing and evaluation of weapons systems — in the official R&D budget.

The new figure emerges from a reassessment by the academy of total US spending on R&D. By excluding non-innovative spending, chiefly at the Department of Defense, the academy comes up with what it considers to be a more accurate measure of expenditure, which it calls Federal Science and Technology (FS&T).

The reassessment follows the publication in 1995 of the report of a panel chaired by Frank Press, a former president of the academy, calling on the administration and the Congress to start counting R&D on this new basis (see *Nature* 378, 426; 1995). The call was ignored, but the academy decided to publish the new measure anyway, in the hope that it will gain currency and authority.

The first assessment finds that FS&T funding in the 1997 financial year (which began on 1 October 1996) will be \$43.4 billion, and that this represents a drop of 5 per cent in spending power since 1994. In the same period, total R&D spending, as measured by the federal government, fell by 2 per cent to \$74 billion.

Press — who was science adviser to President Jimmy Carter — says that the new assessment reveals for the first time the extent to which support for science and tech-



US spending on 'Federal Science and Technology' (FS&T) has fallen more than official R&D figures.

nology has been falling. "The president says that he wants to strengthen R&D, and the Congress says the same," he says. "One can gauge from these figures whether officials in the government and Congress are really doing that."

But he does not accuse Clinton or the Congress of duplicity. "I don't think they realize what all their separate decisions have added up to," he says. The object of the exercise, he adds, is to help both branches of the legislature see what is really happening to research spending.

Press and officials of the academy are now briefing administration and congressional officials on their results. Last week, Press met Frank Raines, the new director of the Office of Management and Budget in the White House, and says that Raines "listened intently" to his message.

The academy's assessment shows a steeper decline than the government's measure of R&D chiefly because the defence department has sharply increased spending on the

testing and evaluation of weapons systems — which the academy does not count as R&D — while cutting back on its actual spending on research and development.

Because the assessment includes this 'real' defence R&D, it also indicates a steeper decline over the past three years than that reported by other assessments which track spending on civil (non-military) research alone.

Press believes that the \$8 billion or so of 'real' military R&D is vitally important; for example, it is the predominant source of support for university research in many disciplines, including computer and materials science and most branches of engineering.

In 1995, the academy proposed a measure for FS&T that would exclude not only military testing and evaluation but also large chunks of non-innovative work at the Department of Energy, and engineering projects at the National Aeronautics and Space Administration (NASA), such as the space station, which costs \$2.1 billion a year.

But at a meeting last year, analysts persuaded academy officials that the space station had to be included as genuine R&D, because its sole official function is as a platform for science.

"This is purely for the purposes of budgetary analysis — it's not a comment on the worth of the space station," explains Norman Metzger, head of the academy's physical sciences section. "If we took the space station out, it doesn't change the overall trends."

The assessment finds that research funding has grown in only two agencies since 1994 — the health department (whose spending is dominated by the National Institutes of Health), which is up by 8.1 per cent, and the National Science Foundation (NSF), which is up by 1.8 per cent.

As measured by the academy, R&D spending is down 11.1 per cent at the defence department, 13.8 per cent at the energy department, and 7.3 per cent at NASA. These figures reflect the real spending power of budgets, after allowing for inflation.

According to some budget analysts in Washington, the academy numbers are not new and could readily be gleaned from official sources and the regular assessments performed by the American Association for the Advancement of Science.

But, unlike the association, the academy concentrates on the big picture, and its assessment has been produced much more promptly than the official one, which is conducted by the NSF.

The academy plans to repeat the exercise twice a year, once in response to the president's budget proposal and again after Congress has appropriated a final budget. It will pay for the exercise itself. **Colin Macilwain**

Human genome centre receives a leg-up

[WASHINGTON] The National Center for Human Genome Research has been raised to the status of an institute within the US National Institutes of Health (NIH), expanding its power and access to funds.

Donna Shalala, Secretary of Health and Human Services, renamed the organization the National Human Genome Research Institute on 14 January, bringing to 18 the number of institutes at the huge biomedical agency.

"We're pleased," says Francis Collins, the institute's director (pictured below). He

says the move recognizes that the genome research centre "has in every way except name been



functioning as a full-fledged institute". Institute status brings increased flexibility with, and control over, research grants, and access to congressional funds earmarked for fighting specific diseases.

Congress funded the centre at \$189 million in 1997. It employs roughly 150 staff and was established in 1989

to carry out the NIH's role in the Human Genome Project. It has also played a key role in policy discussions through its Working Group on the Ethical, Legal and Social Implications of Human Genome Research.

The centre's elevation to institute status initially came in a bill reauthorizing funding for the NIH, which passed the Senate last year. But when the bill was not brought up in the House of Representatives, Shalala took another route, writing to the chairmen of the congressional committees involved with NIH funding, informing them of her intent to elevate the

Science advisers face 'credibility crisis'

[WASHINGTON] A group of leading environmental scientists was warned last week by two senior congressional staff members that a series of politically charged debates in coming months could exacerbate a continuing "crisis of credibility" for scientists who advise policymakers.

The congressional officials also said that scientists should make a point of meeting members of Congress and their staff members to explain not only the benefits of science, but also its limitations in helping to resolve difficult social issues.

The advice came in an unusual hour-long briefing to the executive committee of the Environmental Protection Agency (EPA)'s Science Advisory Board, the congressionally mandated 100-member body of outside experts that reviews research produced by the

agency and its contractors.

The executive committee invited both Republican and Democratic staff members to the briefing, but only representatives of the minority Democratic party were able to attend. They were Michael Rodemeyer, chief Democratic counsel to the House of Representatives Science Committee, and Thomas Sliter, minority staff director for the Senate Committee on Environment and Public Works.

Both said their remarks reflected their personal opinions rather than those of their respective committees. But they agreed that, despite a broad respect for science among politicians, there is "a very disturbing confusion between science and policy" in Congress.

"Congress expects too much of science," said Rodemeyer, and is often puzzled or angry

when scientists cannot deliver a single, clear answer to some difficult environmental question such as how clean-air laws should be written. Scientific uncertainty "may be one of the most difficult concepts for Congress to deal with," said Sliter.

The risk for scientists is that uncertainty becomes expressed in public in an adversarial way, with "duelling experts" giving conflicting testimonies that cancel each other out. According to Rodemeyer, this contributes to a growing perception among politicians and the public that "science really doesn't have anything to contribute to policy debates".

Both said that one solution is for scientists, both in groups and as individuals, to educate congressional members and their staff behind the scenes. "There is no substitute on Capitol Hill for face-to-face meetings," said Rodemeyer.

Sliter predicted that proposed EPA regulations on pollution from ozone and airborne particulates will be a "very hot political issue" in this Congress (see *Nature* 384, 392; 1996). His committee will hold hearings on the subject next month.

Many controversial environmental issues left unresolved by the previous Congress — such as the protection of endangered species and wetlands, and clean water — will also be up for discussion. The debate is expected to be less hostile this year than last. But Republicans are still expected to oppose tighter environmental regulation.

Meanwhile, EPA's Science Advisory Board is struggling over how to handle the controversy that frequently arises from its reports. Several members of the board's Clean Air Scientific Advisory Committee, which was divided in its review of the proposed EPA regulations on ozone and particulates, have publicly expressed doubts about the scientific foundations of the new rules.

In a draft report prepared last month, the subcommittee agreed that board members should always be careful to distinguish between personal views and a committee's view, and should stand behind a report once it is completed.

Meanwhile, as it braces itself for the congressional debate on clean-air laws, EPA also is continuing to work on ways to shore up its scientific credibility. The Office of Research and Development (ORD) — the main producer of basic environmental research in an agency that is primarily geared toward regulation — was given authority last week to review the way in which other EPA offices responsible for air, water and solid-waste and other regulations conduct peer review of their research products. That gives Robert Huggett, the head of ORD and a respected scientist, more oversight of the agency's research.

Tony Reichardt

Staff cuts proposed for energy department

[WASHINGTON] A panel of high-ranking Department of Energy (DoE) officials and outside advisers is calling for a major reduction in the size of the agency's headquarters and field management bureaucracy, as a step towards improved efficiency of the US system of national laboratories.

In a report expected to be made public next month, the department's Laboratory Operations Board says that the agency's current structure, which is widely seen as a left-over from the Cold War, is no longer appropriate in the light of technological advances that have improved long-distance communications. It calls for "appropriate consolidations" between headquarters and field management staff.

"Field operations seem to be largely process-oriented rather than mission-oriented," says the report. It adds that the problem is compounded by the fact that the field offices report to a separate headquarters field management office, rather than to the agency programmes that fund the laboratories. Energy department laboratories report to field offices that are usually nearby, but funding is

provided by programme offices at headquarters.

The report, *R&D Program Management Reviews*, is a follow-up to a *Strategic Laboratory Missions Plan* released by the agency last summer. It is intended to advise programme managers at DoE on whether they are using the right mix and number of R&D performers — namely the laboratories, universities and industry.

Both reports are follow-ons to the February 1995 report by the panel known as the Galvin Task Force, which also recommended a major reduction of the DoE system of laboratory management.

The report of the operations board calls for more planning across DoE programmes, and recommends that the laboratories should be operated more as a system, sharing work when it makes sense and making specialists available for short-term projects, regardless of which laboratory or DoE organization has the need. It says the laboratories should consider combining their management functions to cut costs and improve quality.

For DoE's Office of Energy Research, which operates the department's five non-

weapons multi-programme laboratories, as well as its accelerator and fusion research facilities, the report suggests that maximizing the use of the user facilities of those laboratories should be a high priority.

These facilities, which include synchrotrons, neutron sources and other highly specialized experimental equipment, are available to outside researchers for non-proprietary research. The panel claims that they are responsible for important advances in "pushing the frontiers of discovery in a wide variety of fields".

Arguing that there is "no overarching roadmap" for the future of such facilities, the board urges a broad study into the needs for and strategy surrounding them over the coming 15-20 years. The review, which should be completed by March 1998, should encompass all fields of research covered by the DoE, including high-energy and nuclear physics, condensed-matter and materials science, life sciences and global climate change. It should also consider whether international cooperation is appropriate.

David Kramer

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Santer: Seeking to avoid conflicts of interest on review panels advising European Commission.

Change for Europe's science committees

[PARIS] Jacques Santer, president of the European Commission, said last week that responsibility for scientific advisory committees should be taken away from those departments of the commission whose interests could conflict with public health and consumer protection.

The proposal follows criticism of the way the commission has managed scientific advice in the bovine spongiform encephalopathy (BSE) crisis (see *Nature* 384, 8; 1996). The draft report of an inquiry by the European Parliament into the handling of BSE alleges that the existing arrangements have been a recipe for conflicts of interest.

Speaking before the parliament's inquiry committee on BSE, Santer said that he would ask his fellow commissioners to approve a reorganization under which the commission's scientific advisory committees would be regrouped into a single division of 'product safety'.

Santer suggested that this division might be attached to the directorate for consumer policy (DG 24). He also promised reforms to deal with the way in which members of advisory committees are nominated, and greater openness in the way they operate.

The aim, he said, is to achieve a "desirable" separation between scientific assessment and decision-making. The scientific veterinary committee, for example, would be removed from the powerful agriculture directorate, which has responsibility for drafting legislation and managing markets.

Santer also gave cautious backing to an earlier proposal by Franz Fischler, the agriculture commissioner, to create an independent European inspection agency (see *Nature* 384, 503; 1996).

The creation of a European equivalent of the US Food and Drug Administration (FDA) "should not be excluded", he said. However, in practice, Santer's proposed agency would be a far cry from a European FDA, as it would only have a staff of about a hundred inspectors.

Declan Butler

AP

Britain plays it cautious on animal-human transplants

[LONDON] Britain has announced a temporary moratorium on xenotransplantation — the use of animal tissue for human surgery — in the light of continuing uncertainty about its safety, including the dangers of transferring viruses from animals to man.

The decision was announced last week in response to the report of a panel set up to study the ethics of xenotransplantation. The report drew particular attention to the potential threat of retroviruses, the genes for which could lie unexpressed in the genome of an animal whose organs are being used.

The most immediate impact of the decision could be a delay in clinical trials of the use in human transplants of pig hearts that have been genetically engineered to reduce the risk of rejection.

The ethics panel, chaired by Ian Kennedy, professor of medical law and ethics at Kings College in London, concluded that there were no ethical reasons why such experiments — and eventually operations — should not eventually be carried out, echoing other reports including that from the US Institute of Medicine (see *Nature* 382, 197; 1996).

But it highlighted a number of issues which, it said, need to be addressed before such trials should go ahead. In addition to the virus threat, for example, it said a comprehensive statutory framework of regulation should be put in place to protect the interests of patients.

In response, Stephen Dorrell, the health secretary, announced last week that not only had the government accepted the panel's judgement on the scientific and ethical

aspects of xenotransplantation, but it had also agreed to introduce legislation to ensure its proposals are legally enforceable.

Until the new legislation is passed, all research in the field will be monitored by a so-called Xenotransplantation Interim Regulatory Authority. Dorrell announced that this committee will be chaired by Lord Habgood, a former Archbishop of York who trained as a pharmacologist and is widely respected for his writings on the relationship between science and religion.

The need for a regulatory framework sharpened 18 months ago with the announcement by a Cambridge-based biotechnology company, Imutran, that it had successfully completed trials of the transplantation of transgenic pig hearts into monkeys (see *Nature* 377, 185; 1995).

It has also been stimulated by increased knowledge about the nature of some of the potential difficulties, including the possible transmission of retroviruses. A team at the Institute for Cancer Research in London, for example, has been able to show that the genetic coding for two C-type retroviruses can be transmitted from pig tissue to human tissue, and that one of these is subsequently expressed in the human tissue.

Scientists working with Imutran and other companies are confident that the safety problems will not prove insurmountable. But it will be for the interim regulatory authority — or whatever succeeds it — to decide whether clinical trials can be carried out on humans before a complete picture of the potential dangers has been worked out. □

'German politicians break research pledge'

[MUNICH] The heads of Germany's leading research organizations last week accused politicians of breaking a commitment to protect research against the pressures caused by the country's economic difficulties.

The scientists issued a statement arguing that neither the economic recession nor the cost of meeting criteria for a unified currency within the European Union justifies reducing investment in education and research, which are essential for economic recovery. It was sent to the federal research minister, Jürgen Rüttgers, and the prime minister of each German state.

The statement, *Priorities for the Future*, expresses a widespread disillusionment with national politics among German scientists. Its signatories include Wolfgang Frühwald, president of the Deutsche Forschungsgemeinschaft, and Hubert Markl, president

of the Max Planck Society.

Despite frequently repeated promises by senior politicians, including Chancellor Helmut Kohl, that Rüttgers' ministry of education and research — which only three years ago was heralded as the 'ministry of the future' — would be selectively protected from financial cuts, it has suffered much greater cutbacks this year than other federal ministries (see *Nature* 384, 399; 1996).

The statement from the scientific leaders also argues that universities should be released from the constraints imposed by the federal 'framework' law (*Hochschulrahmengesetz*), whose reform is currently being debated (see *Nature* 384, 204; 1996). It calls for the law to be abandoned so that universities could choose their own students and set their own rules, allowing for real competition between them.

Alison Abbott

The 's' in Unesco seeks out a new role

[PARIS] The United Nations Educational, Scientific and Cultural Organization (Unesco) has set up a 50-strong International Scientific Advisory Board to give working scientists a greater say in a planned reform of the way the agency supports science.

The board, which met in Paris for the first time this week, is made up of eminent scientists from industrialized and developing countries. They include Nobel laureates Carlo Rubbia, Ilya Prigogine, Jean Dausset, Georges Charpak, Paul Berg and Werner Arber, who chairs the board and who is also president of the International Council of Scientific Unions (ICSU).

According to Federico Mayor, Unesco's director general, the board's main role will be to advise on reform of the agency's scientific activities, and also to help prepare the agenda for a UN world science summit which Unesco plans to hold in 1999 in collaboration with ICSU.

Mayor says that the beginning of the new millennium is an appropriate time to put the 's' back in Unesco, alluding to the fact that science was not included in the original title. Although 'Uneco' was eventually changed to 'Unesco' — after the British scientists Joseph Needham and Julian Huxley had argued that science was part of culture — many argue that science has since become the weakest area of the agency's activities.

"To see Unesco's role in science you have to go back in history," says David Ottoson, secretary general of the International Brain Research Organization, and a member of the new advisory board, alluding to Unesco's heyday in the early post-war years, when it was instrumental in creating CERN, the European Laboratory for Particle Physics.

Useful but less visible work

Since then Unesco's science programme has been "very weak", says Ottoson. But he says it has nevertheless done much useful, though less visible, work through its support for ICSU. He points out that science has been a poor relation to education within the agency, and argues the need to alter this situation.

Others say that Unesco's impact on science is doomed to remain small, given its administrative structure and relatively low funding compared to national research budgets. Unesco's budget for 1996 and 1997 is \$518.4 million — of which \$84.1 million is dedicated to support for science — plus \$290 million from other UN agencies and member states.

This is about equal to the budget of a large university, and a drop in the ocean compared with the needs of developing countries alone. The budget is much less than is needed to implement comprehensive science programmes in Unesco's 185 member states.

The funding problem is compounded by the continued absence from membership of Unesco of both the United States and the United Kingdom.

The agency seems to have woken up to its limitations. In *Unesco, the Will to Reform*, a strategy document published last year, it pointed out that since 1988 it had trimmed its number of programmes from 392 to 139. The aim was to meet the demand of member states that it "avoid channelling money into a variety of projects having little or no impact, and concentrate its activities on fewer but better conceived programmes".

The agency also intends to stop directly supporting research projects apart from small amounts given to projects in poor countries, which often demand a *juste retour* on their Unesco subscription, according to



Mayor: values Unesco's role on ethical issues.

Maurizio Iaccarino, an Italian molecular biologist who was appointed assistant director general for science last year.

Unesco's task should instead be to arrange technical evaluation of projects in developing countries, he says, and to provide qualifying projects with a Unesco 'label' that will help to attract other funding.

This 'hands-off' approach also reflects the fact that staff cuts at Unesco have curbed a tendency to create new bureaucratic structures to manage new projects. Staff numbers have fallen from more than 3,000 in 1987 to around 2,200 today, while the science division has just 270.

Getting outside scientists involved in setting strategy through the new advisory board is one way to compensate for Unesco's lack of staff and professional expertise. Panels of outside scientists have proved their usefulness in Unesco's well respected inter-governmental commissions, such as those that coordinate international research on oceanography, hydrology and geology.

Staff cuts have taken their toll on morale. One observer claims that many of the best people have now gone, and that the cuts have undermined the agency's intellectual capacity. "It's a desert here," claims one official at Unesco's Paris headquarters. "At six o'clock, everyone has gone home except the cleaners."

Besides adjusting Unesco's administration to financial realities, the main challenge still facing Mayor is to adapt the organization's goals to the contemporary world. Mayor refers to the need "to get back to our

constitution, which says we must build peace through transfer of knowledge". But as Julian Huxley, Unesco's first director general, once remarked, the constitution amounts to "a starry-eyed hope rather than a practical guide to action".

Critics argue that top-down organizations such as Unesco are increasingly irrelevant in a post-Cold-War world marked by decentralization — exemplified by the Internet — and a shift in research goals from strategic and ideological ends to innovation and economic competitiveness. One critic points out that the emerging economies of Asia developed their technological economies independently of Unesco.

Similarly, many large research organizations see no need to go through Unesco. Patricia Psuchitani, from the Africa Division of the US National Science Foundation, says that it has no contact with the agency, preferring to establish cooperative research programmes through bilateral and multilateral agreements.

A potentially unique role

But some hardened critics of Unesco nonetheless agree that in principle it remains uniquely placed as the only international intergovernmental agency with an explicit remit to promote fundamental science. They say it has a vital philosophical role in the "battle of ideas" to persuade developing countries of the need to invest in a science base if they are to profit from aid programmes and technological progress. "It would be a great pity" if Unesco were to be dissolved, says one critic.

Mayor sees Unesco's philosophical role as an international forum for debate on the ethical issues surrounding scientific progress as a major plank in the agency's future. Indeed, its International Bioethics Commission, with 50 members from developing and industrialized countries (one-third of whom are scientists), has been widely acknowledged as a success.

The commission has prepared a draft of a "universal declaration on the human genome and human rights" which will be submitted for approval to the Unesco General Assembly later this year. Mayor would like to extend this approach to other areas, by creating similar forums of scientists, lawyers, intellectuals and other representatives of society to discuss at a world level the social aspects of issues such as energy, information technology and biotechnology.

"Science is a political issue and part of the decision-making process, and we need to take into account the social dimension," says Mayor. "This is the main contribution Unesco can make."

Declan Butler

Canada calls in US nuclear power experts to boost performance

[QUEBEC] In what its president has called "a humbling experience", the Canadian public utility that was formerly considered a world leader in nuclear power has had to hire US experts to improve the performance of its nuclear power plants.

Carl Andognini, who has 30 years' experience with major US utilities, is to take over as head of Ontario Hydro's nuclear division by the end of this month, assisted by six other US nuclear engineers. A spokesman for the company said it felt that the kind of expertise it needed did not exist in Canada.

The US team was hired on the orders of Allan Kupcis, president of Ontario Hydro. "We've got into believing our own headlines that we're at the top of the world," says Kupcis, referring to boasts during the 1980s that the utility's Candu heavy-water reactors were achieving maximum output for more than 80 per cent of the time. The average has dropped to just under 70 per cent.

Nuclear power accounts for at least 60 per cent of Ontario Hydro's energy output. But over the past ten years the efficiency of its plants has steadily diminished. The Candu reactors are designed and sold by Atomic Energy of Canada Ltd (AECL).

Officials at the design company and the utility say that the problems are not the fault of the technology, but arise from a lack of management expertise that has led to sloppiness in maintenance. For example, a piece of wood was once left in a reactor during repairs. By the time it was retrieved it had disintegrated, causing a disruption to power supplies. On another occasion a lead blanket was left behind by workmen.

A spokesman for the Atomic Energy Control Board, the nuclear-power regulating agency, says the board has been "badgering" Ontario Hydro for some time to improve its safety practices. The Pickering plant, for example, has been given only a six-month licence to operate.

Elsewhere in Canada, there have been mechanical problems with reactors. Reactors at Point Lepreau in southern New Brunswick and Gentilly in Quebec have experienced serious corrosion in the feeder pipes that carry heavy water from fuel channels to boilers to produce steam.

The corrosion seems to be caused by a combination of temperature, mass velocity and iron concentration in the water. These reactors use boiling water. Although there

has been some corrosion in the Ontario reactors, which operate at a lower temperature, the problem was not expected to become serious for 20 years.

AECL engineers believe they know how to reduce the corrosion. But they are awaiting results of experiments at Chalk River laboratories, in Ontario, for a definitive answer. Meanwhile, they will provide directives for interim changes by the end of this year.

Candu reactors have been sold to Argentina, South Korea, China and Romania. Argentina's suffers from corrosion, but less than at Point Lepreau and Gentilly, for reasons that are not completely understood. South Korea's reactor is experiencing a more serious problem. Romania's reactor has only just started operation, and has not so far developed any problems.

AECL engineers hope that the corrosion problem will not affect foreign sales. "We've got solutions and we can change materials," says one. "In reactors that we have, we've changed the composition of the steels, so we can avoid the problem in that way." He says that any change to operating conditions will be applied abroad.

David Spurgeon

Japan fishes for more space launches

[TOKYO] Japan plans to renegotiate an agreement with fishermen in the vicinity of its two space-launch centres in southern Japan — almost doubling its annual payments to fishing cooperatives of ¥400 million (US\$3.5 million) — to allow launches on a greater number of days each year.

The move is part of a bid to increase the number of launches for its two space agencies for both commercial and scientific missions. There will also be an expansion of launch facilities for the National Space Development Agency (NASDA) which will be used for both commercial and government-sponsored launches.

The Science and Technology Agency (STA) will soon start negotiations with cooperatives in the prefectures of Kagoshima, Miyazaki and Oita in the southern island of Kyushu, and Ehime and Kochi in the neighbouring island of Shikoku. These are relatively close to the launch centre of the Institute of Space and Astronautical Science (ISAS) on the coast of Kagoshima and that of NASDA, just off the Kagoshima coast on Tanegashima island.

Fishermen claim that launches could damage fishing grounds, and the present agreement with them limits each space agency to making launches during two periods of 45 days — one in winter and one in summer. This effectively restricts each

agency to a maximum of two launches a year.

The payment to the fishing cooperatives is made by STA as part of the agreement to allow the 90-day launch period. The science agency has budgeted ¥700 million — almost twice the current amount — for the fishermen in its fiscal 1997 budget, which begins on 1 April, to expand the period.

Both the STA, which funds NASDA, and the Ministry of Education, Science, Sports and Culture (Monbusho), which oversees ISAS, want a longer period. NASDA has just begun building a new ¥25-billion (US\$217-million) launch pad on Tanegashima, due for completion early in 2000. Along with the existing pad, this will allow launches in rapid succession.

Completion of the new pad is timed to coincide with the first launch of a more economical version of NASDA's H-2 rocket, the H-2A (see *Nature* 378, 527; 1995). The H-2A will be used by both NASDA and Rocket Systems Corporation, a private consortium of 73 Japanese aerospace companies, banks and insurance companies which was set up in 1990 to sell launches of the H-2 (see *Nature* 345, 191; 1990).

The corporation has agreed with Hughes Space and Communications International of the United States to make 20 launches, in a five-year period from 2000, carrying commercial satellites.



NASDA also expects to increase its number of launches to launch and service the Japanese module for the international space station which is due to start operations early next century. And both NASDA and ISAS have ambitions to carry out scientific interplanetary missions that will require greater flexibility in launch periods (see *Nature* 370, 405; 1994).

STA is not prepared to say what launch period it hopes to agree with the fishermen, as that is "a matter for negotiation". An agency official stresses that the ¥700 million will not be paid in cash, and it is not "compensation". Rather, it is intended to assist fishermen by providing new facilities such as improved refrigerators.

David Swinbanks

UK research councils sharpen spending on strategic goals

[LONDON] About 70 per cent of the research funded through Britain's seven research councils is carried out in areas identified by the Technology Foresight programme, compared to 45 per cent four years ago when the programme was introduced, according to the Office of Science and Technology.

The shift in orientation was revealed last week in details of the allocation of next year's science budget. This contained few surprises, with a previous decision to maintain overall spending roughly constant — the 1997–98 budget of £1.33 billion is 1.4 per cent higher than the current year — being reflected in virtually level funding for all the councils.

The only major new initiative is a new programme of research into transmissible spongiform encephalopathies. A total of £17 million will be allocated to this over the next three years, and the Medical Research Council has announced a grant of almost £1 million to John Collinge of St Mary's Hospital in London to develop a new unit dedicated to human prion diseases.

Pressure on the Particle Physics and Astronomy Research Council has been significantly reduced by a cut of £5 million in Britain's contribution to CERN.

Austrian privatization brings funding windfall

[MUNICH] Austrian researchers will be among the major beneficiaries of a ÖS17-billion (US\$1.6-billion) windfall received by the government following the long-planned privatization of Creditanstalt-Bankverein (CA), the state-run bank, which was announced last week. The government will make available ÖS1 billion for new research programmes during this year, to be followed by a further ÖS1 billion over each of the following two years — provided it is satisfied that the first round of funding is wisely spent.

Half of the cash will be distributed by Austria's two research funding agencies, for pure and applied research. The other half will be used to build up Austria's centres of excellence, with particular emphasis on promoting their technology transfer capabilities.

Consensus meetings may tackle bioethics

[PARIS] François d'Aubert, the French secretary of state for research, last week proposed that the national bioethics committee should extend its work to include 'conferences of deliberation' modelled along the lines of the consensus conferences held in many Scandinavian countries: panels of lay

people vote on issues such as the desirability of genetically modified foods after having consulted widely with experts.

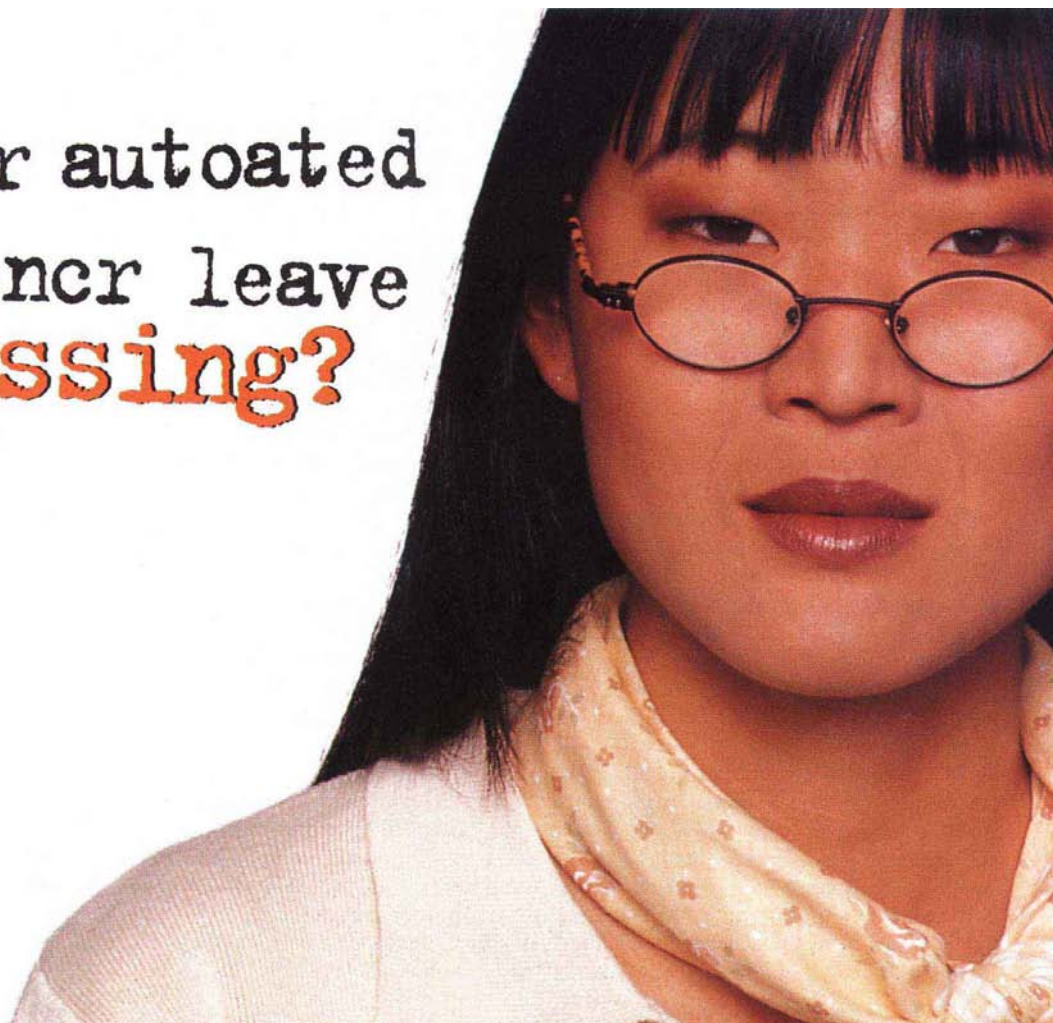
D'Aubert praised the ethics committee itself — which is made up of scientists, jurists, philosophers and other 'sages' — for having pioneered dialogue between science and wider society. But he said that involving lay people more in such debates would help to increase public confidence in the decision-making process, and invited the committee, the French Academy of Sciences and the Parliamentary Office of Technology Assessment to put the idea into practice.

'No difference' in chronic illness in Gulf War troops

[WASHINGTON] An analysis of two voluntary registries of US troops engaged in the Gulf War has found no difference in the incidence or type of self-reported chronic illness between those who may have been exposed to nerve gas when an Iraqi munitions depot was destroyed in March 1991 and those not in the vicinity, according to findings due to be presented to a congressional committee on 21 January.

The *Washington Post* said last weekend that investigators at the Departments of Defense and Veterans Affairs reached this conclusion after examining the medical files of several thousand soldiers who were near the depot

Does yur autoated
DNA seqencer leave
u **guessing?**



Pros and cons of foreign genes in crops

Sir—In a leading article you expressed your scepticism about leaving antibiotics genes in agricultural transgenic plants (*Nature* 383, 559; 1996). This view was criticized by Abigail Salyers (*Nature* 384, 304; 1996), who believes that it may detract from the more important fight against “the continued abuse and overuse of antibiotics by physicians, over-the-counter sale of antibiotics, and use of antibiotics in animal feed”. These positions should not, however, be pitted against each other.

It is easy to understand why there should be restrictions on the direct use of antibiotics in the human environment. It may be less obvious why regulating bodies should take a critical view of harmless antibiotics genes in agricultural plants. These genes are needed to introduce the transgenic characters into the plants, but are not vital for the growth of the plants in the field. Methods can therefore be designed to remove them selectively after they have finished their task.

There are two reasons why breeders should be asked not to leave such antibiotics genes in their products. The first is that all crop plants in the future will be transformed not only once but many times. If every transformation leaves another antibiotics gene in the plant, then it will soon become difficult for the plant breeders to find new ‘harmless’ antibiotics genes to use. The second reason has to do with what the public expects from the new gene technology. Commercial plants with transgenic properties will become generally accepted only if the arguments in favour of their use are strong and convincing. The best argument for them is, undoubtedly, the precision by which they have become altered, by comparison with, for example,

classical plant breeding. But if gene technology is to be presented as a clean technology, then it must be clean. From personal experience in the Swedish Gene Technology Board, I know how difficult it is to argue for a new crop variety containing an interesting character if it also carries some antibiotics gene of no relevance to the needs of the farmer or the consumer.

So setting high standards for new transgenic plant varieties is not only a question about human health. It is also a way to protect a vital new technology against shortsighted uses that may later lead to severe setbacks.

Bengt O. Bengtsson*

Department of Genetics,
Silvegatan 29, S-223 62 Lund, Sweden
e-mail: bengt_olle.bengtsson@gen.lu.se

*Address until June 1997: 32 rue de Chazelles, F-75017 Paris, France.

Sir—Your News story “Europe agrees a compromise on food labels” (*Nature* 384, 502–503; 1996) pointed out that consumer, food industry and environmental groups have weighed in with opinions on the newly proposed compromise on labelling of genetically modified foods in Europe. Conspicuously absent from the article was any mention of views from within the science community about an issue that has fundamental scientific components.

Although disclosure of the contents of food is an important issue, the potential categorization of food into genetically modified and non-modified groups warrants more discussion.

For example, triticale, a polyploid plant containing full copies of both rye and wheat genomes, was developed 60 years ago and is now grown on more than a million hectares in Canada, Mexico and eastern Europe.

Modern plums contain chromosomes from cherry plums and blackthorn. Russian wheat has genes from both rye and wild wheat grass, and French plant breeders have introduced fungal eyespot disease-resistance genes from goat grass into French domestic wheats. This is a sampling of a much longer list illustrating that many present-day crops used to produce food for humans have for years contained foreign genes, and could arguably be considered novel foods, without the application of recombinant DNA technology.

Newer transformation technologies clearly expand the range of foreign genes that can be introduced into crops, but the public debate centres principally on food safety issues. The perceived novelty of ‘foreign’ genes in foods may be responsible, at least in part, for concern about safety for human consumption and for the environment.

The interspecific genetic modification of foods is clearly not inherently new. Those writing regulations relating to a scientific determination of safety of foods should give significant weight to input from the scientific community in addition to concerns of other interests. The more the focus is kept on safety of the product for humans and the environment, with decisions being made on the basis of the soundest scientific findings rather than on novelty, the better the result should be for consumers and the environment.

Donald R. Ort

(President, American Society of Plant Physiologists)
USDA/Agricultural Research Service
and University of Illinois,
Urbana, Illinois 61801-3838, USA
e-mail: d_ort@uiuc.edu

Climate of mistrust

Sir—In his review of Andrew Rowell’s book *Green Backlash*, Julian Morris appears to have constructed a straw man and set about burning it (*Nature* 384, 325; 1996). Morris is eloquent and correct in his observations on the scientific method and the importance of rigorous criticism in science. But that is not the subject of *Green Backlash*.

My reading of the book was as an attempt, *inter alia*, to examine the way in which scientific knowledge is translated into a policy response and how this is open to abuse. As Morris says, “the possibility always exists that an alternative, better, theory might be found”. The climate-change negotiations have been bedevilled

by well-organized lobbyists who have sought to use this argument as a reason to avoid or delay action. But policy-makers have to act, and cannot delay judgements in the face of uncertainty. Even a decision to do nothing is to act as if you believe there is no cause for concern. All decisions require a working hypothesis, and, for climate change, Rowell is right to argue that the basis of policy should be that it is happening and is dangerous. This is not, however, to claim that the working hypothesis is an incontrovertible truth.

In fact, the proper application of scientific method strengthens the case for strong action to respond to climate change. This is because the alternative hypotheses that attempt to explain how anthropogenic greenhouse gas emissions do not lead to

rising atmospheric concentrations and how these do not increase radiative forcing and surface temperature are very poor and challenged by plenty of confounding evidence.

These other hypotheses have so little credibility that their proponents, of whom Morris is one, have had to form the European Science and Environment Forum in order to publish them. It is not surprising he did not like *Green Backlash*, given that its purpose is to unmask such organizations and examine their motives.

Clive Bates

International Institute for
Energy Conservation — Europe,
1–2 Purley Place,
London N1 1QA, UK

Too quiet to hear a whisper

P. Jung and K. Wiesenfeld

Add some noise, and many dynamic systems respond to weak signals more strongly. Some sort of threshold process was thought to be necessary for this effect, but a new model removes the restriction.

Fluctuations usually destroy order, yet there are situations where noise plays a surprisingly constructive role. This realization has triggered a lot of excitement in recent years, most notably in the study of the phenomenon of stochastic resonance, which is observed in experiments including ring lasers, superconducting magnetometers and sensory neurons. Now it seems that stochastic resonance is even more common than previously suspected, according to a theory by Sergey Bezrukov and Igor Vodyanoy reported on page 319 of this issue¹, with possible implications for systems ranging from mesoscopic electronic devices to signal transduction in ion channels through cell membranes.

The basics of stochastic resonance were laid out in the early 1980s, with speculation that noise might be responsible for the apparent periodicity of ice ages (the Milankovitch cycles) by amplifying tiny periodic variations in the Earth's orbit^{2,3}. There followed a wealth of theoretical and experimental work, leading to a well-settled understanding of the effect. The conditions that lead to stochastic resonance are fairly simple and robust, which is why it has been observed so readily in so many different experiments.

Over time, the original notion of stochastic resonance has widened to include a number of different mechanisms. The unifying feature is that noise can improve a system's sensitivity to weak signals, such as periodic inputs. One measure of sensitivity is the out-

put signal-to-noise ratio, and a signature of stochastic resonance is when this ratio passes through a maximum as a function of the applied noise intensity. The earliest research focused on bistable systems (a class that includes the ice-age model), but has since been expanded to include excitable systems⁴ that have a single rest-state. Study of these systems was motivated by the potential relevance of stochastic resonance to neuronal dynamics.

Simple cartoon versions of excitable systems known as threshold detectors⁵ have been considered intensively because they are accessible to full analytical treatment. In these models, the detector responds (by sending out a pulse, for example) whenever the sum of the signal and the noise crosses a threshold. A crucial ingredient for a system to show stochastic resonance was thought to be the existence of a potential barrier, or a threshold of some sort. Bezrukov and Vodyanoy, however, say otherwise.

They consider a theoretical device which spits out a random spike train with a constant spike rate. In their model, this rate depends exponentially on an external input, such as a voltage. So as the input varies in time, the spike rate changes: the spiking pattern thus encodes, by its inhomogeneity, the input signal (Fig. 1). The input is the sum of a weak sinusoidal signal and external noise. A key aspect of the model is that — in contrast to earlier work on spike trains generated by excitable detectors — there is no threshold

or potential barrier. That is, an arbitrarily weak signal affects the spike train even in the absence of external noise.

Bezrukov and Vodyanoy calculate the signal-to-noise ratio of the outgoing spike train. The main result is that the ratio goes through a maximum as the external noise strength is increased, a signature of stochastic resonance. In order to carry through the analysis fully, they make some assumptions: that there are no spike-spike correlations; that the amplitude of the sinusoidal signal is small; that the spike rate is exponentially dependent on the inputs; and that the averaging over the inherent randomness and external noise can be performed separately (that is, there is no causal relationship).

In real systems these points (especially the last one) will need investigation, but the model is certainly plausible. Indeed, Fig. 2 shows data supporting it for one of the candidate systems listed by Bezrukov and Vodyanoy (B. Meadows and W. Ditto, personal communication). The output signal-to-noise ratio for a strongly reverse-biased electronic diode is plotted against input noise intensity. In a diode, the output current consists of clusters of charge carriers with inherent randomness, and this output can be viewed as a spike train. The current is the mean flow rate of charge carriers, and depends exponentially on the applied voltage. Although it remains to be explored how far the model assumptions are fulfilled for this particular system, the data clearly show the stochastic resonance peak.

By doing away with a sharp threshold, Bezrukov and Vodyanoy show that stochastic resonance applies to a new class of systems. The new requirement is that the spike rate depends exponentially on the input, but that is a fairly common property. For example, the rate at which electrons boil off a conductor (thermionic emission) depends exponentially on the surface potential, and the current through ion channels is similarly sensitive to a voltage difference across the cell membrane. In fact, an earlier experiment⁶ by Bezrukov and Vodyanoy on alamethicin ion channels in a lipid bilayer was a motivating factor for this newer theoretical work.

It is intriguing to speculate to what extent this property has a function: is stochastic resonance widely exploited in biological systems to detect weak signals? This remains very much an open question, but the step taken by Bezrukov and Vodyanoy is a further temptation to explore the role of noise in signal transduction. □

P. Jung and K. Wiesenfeld are in the School of Physics, Georgia Institute of Technology, Atlanta, Georgia 30332-0430, USA.

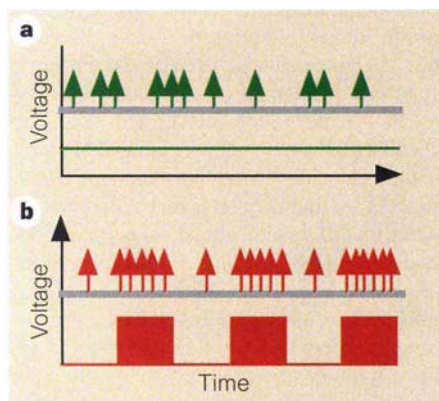


Figure 1a, A typical spike train, generated with constant input, compared with (b) a spike train generated by a periodic rectangular input. The properties of the input signal are reflected in the spike pattern.

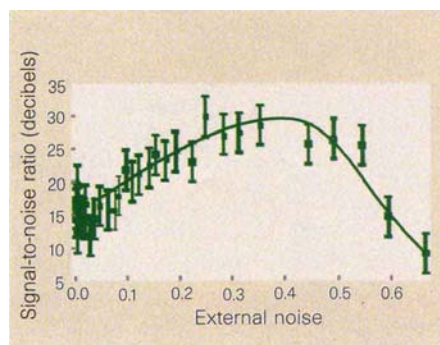


Figure 2 A strongly reverse-biased p-n silicon diode is driven by a weak sinusoidal voltage. With no external noise, the output signal-to-noise ratio is very small. Adding gaussian noise to the weak input, the signal-to-noise ratio increases to a maximum at a finite noise intensity.

1. Bezrukov, S. & Vodyanoy, I. *Nature* **385**, 319–321 (1997).
2. Benzi, R., Sutera, A. & Vulpiani, A. *J. Phys. A* **14**, L453–L457 (1981).
3. Nicolis, C. *Tellus* **34**, 1–9 (1982).
4. Longtin, A. *J. Stat. Phys.* **70**, 309–327 (1993).
5. Gingl, Z., Kiss, L. B. & Moss, F. *Europhys. Lett.* **29**, 191–196 (1995).
6. Bezrukov, S. & Vodyanoy, I. *Nature* **378**, 362–364 (1995).

The oldest whodunnit in the world

Bernard Wood

Modern human technology had humble beginnings. The earliest signs of this are the ability to select a suitable pebble from a stream bed, and to strike it with an appropriate stone to produce either sharp-edged flakes or a crude chopping tool. On page 333 of this issue, Semaw *et al.*¹ present new evidence from Gona in Ethiopia (Fig. 1), which pushes the date for the onset of tool manufacture back to at least 2.5 million years (Myr) ago. And last month in the *Journal of Human Evolution*, Kimbel *et al.*² reported that at the nearby 2.3-Myr-old site at Hadar, similar-looking stone tools were found in "close association" with a jawbone from one of the earliest examples of the genus *Homo*. But how secure is the claim that hominid technology is so deeply rooted in the past, and how justified is the assumption that the first technologists were members of our own genus?

The assertion that stone-tool manufacture goes back 2.5 Myr is based on evidence from the Gona River, which is in the Awash Valley in Ethiopia. The Awash Valley is best known for the collections of fossil hominids that have been found at Hadar, to the north-east of Gona, and at the Middle Awash, which is to the south. Thus far, work in the Awash Valley has concentrated on strata that are between 2.9 and 3.9 Myr old. Although these efforts have yielded an impressive collection of an early hominid species known as *Australopithecus afarensis*³⁻⁵, even after many years of field work there have been no signs of stone tools in these earlier strata.

There is no doubt that the two teams have recovered genuine and remarkably sophisticated artefacts. Of the several thousand tools that were discovered by Semaw *et al.* at Gona,

more than a thousand were found *in situ* at two excavations. Kimbel *et al.* recovered a more modest haul from Hadar (locality A. L. 666) — just 34 artefacts — but 14 of these were from an excavation. The geological context of the artefacts at both sites is reassuring: at neither location is there any evidence that the tools might have been made more recently and then deposited in the beds of stream channels that had cut into the older layers. The sediments are too fine-grained to be channel deposits, and the edges on the tools are fresher than they would have been if they had been rolled in stream beds. Stone-on-stone percussion can occur naturally, but it is inconceivable that random impacts could mimic the uniform flaking patterns of many of these artefacts.

Archaeologists use the term 'industry' for distinctive collections of stone tools, and both the Gona and the Hadar artefacts have been assigned to the 'Oldowan industry'. This was the name given by Mary Leakey to the 1.8-Myr-old stone tools that she recovered from Bed I at Olduvai Gorge in Tanzania. Oldowan-like artefacts have been found at sites elsewhere, but until the new discoveries at Gona, the earliest evidence came from the Lokalalei site at West Turkana⁶ which, like A.L. 666 at Hadar, is around 2.3 Myr old.

Stone tools of this antiquity cannot be dated directly — their ages have to be inferred from the strata that contain them. Semaw *et al.* used two dating methods and they relied on the ability to trace distinctive, reliably dated, strata from site to site and from one locality to another. A stratum below the two excavations is apparently equivalent to the BKT-2L₁ tuff-derived layer from Hadar, and single-crystal argon/argon

(Ar/Ar) dates confirmed that it results from a volcanic eruption that took place around 2.9 Myr ago¹. A date of about 2.5 Myr from another altered tuff (the AST-2.75), which occurs just above the excavations, and the identification of the 2.6-Myr-boundary between the Gauss and the Matuyama magnetozones just below the artefacts, bracketed them as being manufactured between 2.6 and 2.5 Myr ago.

The key to the dating of the hominid (A. L. 666-1) and the stone tools from Hadar is an ash-derived layer, designated BKT-3, which overlies similar sediments nearby. The BKT-3 layer is contaminated with much older volcanic ash which has a different chemical signature. Single crystals have been recovered from the dominant population of more recent grains, and Ar/Ar dating from these suggests that the A. L. 666 locality is around 2.3 Myr old.

The A. L. 666-1 hominid maxilla (jawbone) is remarkably well preserved and it retains enough features for Kimbel *et al.* to be confident that it does not belong to *Paranthropus boisei*, which is known from this time period⁷. Nor does the A. L. 666-1 maxilla resemble the upper jaws of the earlier *Australopithecus afarensis*. Only one species of early *Homo* — *Homo rudolfensis* — has been identified in sediments which may be as old, or even older^{8,9}, than those that have yielded A. L. 666-1. However, Kimbel and his colleagues are sensibly cautious and they allocate it to 'early *Homo*' with no species tag.

If we accept that the maxilla belongs to early *Homo*, does this mean that early *Homo* made the artefacts that apparently come from the same horizon? 'Guilt by association' of this sort — namely that the maxilla and the artefacts derive from the same 3.5-metre-thick fossiliferous horizon — is simply not justified. Even when hominid remains have been found with artefacts and broken bones on so-called 'living floors', such as those at Olduvai Gorge¹⁰, unless we can be certain that there was only one hominid species at that time then there can be no guarantee that a particular hominid species fashioned the artefacts.

In my view, what is more significant about the evidence from Gona and Hadar is something that Semaw *et al.*¹ point out in passing — that there was technological stasis in the 'Oldowan industrial complex' for approximately a million years, between around 2.5 and 1.5 Myr ago. So if any hominid group is to be linked with the manufacture of the Oldowan industry then, logically, it should be one that spans the same time range.

No early *Homo* species satisfies this criterion, as evidence for the Oldowan persists long after *Homo rudolfensis* apparently disappeared from the hominid fossil record¹¹. The main morphological break in the *Homo*



Figure 1 Early workshops: the sites in East Africa discussed in the text. Semaw *et al.*¹ report that they have recovered the oldest known stone tools (2.5–2.6 Myr old) from Gona in Ethiopia; previously, the oldest tools (2.3 Myr old) were those recovered from Lokalalei in Kenya. Another collection of stone tools has been discovered by Kimbel *et al.*² at Hadar in Ethiopia. These were found in "close association" with hominid remains. So, the question now is, who made the tools?

fossil record apparently occurred just less than 2 Myr ago, with the emergence of *Homo ergaster*¹². So if we want to link *Homo* with any particular behavioural innovation, we should seek it in the archaeological record of around 2 Myr ago¹³. There is one hominid group that fits the criterion, and that is *Paranthropus*. There is evidence that *Paranthropus aethiopicus/boisei* existed in the Omo region of Ethiopia across the same approximate time range (2.5–1.5 Myr ago). Although early on there was an episode of rapid dental evolution within the lineage⁷, it has otherwise shown a remarkable degree of morphological stasis, retaining a wide face and large chewing teeth throughout. The apparent conformity between a consistent dietary adaptation and technological stasis is powerful circumstantial evidence to link *Paranthropus* with the earliest artefacts.

We know little about the conjunction of factors that prompted the onset of stone-tool manufacture. What were the relative contributions of hand-eye coordination, manual

dexterity, conceptual facility and opportunity? We certainly know too little to bar *Paranthropus* from consideration¹⁴, or to assume that *Homo* must be the culprit. In *Paranthropus* we have a plausible suspect that had access to the 'weapon' and the 'opportunity', but what was the 'motive'? □

Bernard Wood is in the Hominid Palaeontology Research Group, The University of Liverpool, PO Box 147, Liverpool L69 3BX, UK.

1. Semaw, S. et al. *Nature* **385**, 333–336 (1997).
2. Kimbel, W. H. et al. *J. Hum. Evol.* **31**, 549–561 (1996).
3. Kimbel, W. H., Johanson, D. C. & Rak, Y. *Nature* **368**, 449–451 (1994).
4. Clark, J. D. et al. *Nature* **307**, 423–428 (1984).
5. White, T. D. et al. *Nature* **366**, 261–265 (1993).
6. Kibunjia, M. J. *Hum. Evol.* **27**, 159–171 (1994).
7. Wood, B. A., Wood, C. & Konigsberg, L. *Am. J. Phys. Anthropol.* **95**, 117–136 (1994).
8. Hill, A. et al. *Nature* **355**, 719–722 (1992).
9. Schrenk, F. et al. *Nature* **365**, 833–836 (1993).
10. Leakey, M. D. *Olduvai Gorge* Vol. 3 (Cambridge Univ. Press, 1971).
11. Wood, B. A. in *Species, Species Concepts and Primate Evolution* (eds Kimbel, W. H. & Martin, L. B.) 485–522 (1993).
12. Wood, B. A. *Koobi Fora Research Project* Vol. 4 (Oxford Univ. Press, 1991).
13. Roche, H. *Ossa* **14**, 97–98 (1989).
14. Susman, R. L. *Science* **240**, 781–784 (1988).

Asteroids

Mantles were battered to bits

Clark R. Chapman

A new view is emerging of the asteroid belt as a mere vestige of a much vaster population that had a violent, destructive early history. This may explain the startling shortage of asteroid mantle material in meteorites found on Earth — a problem that has become even more severe according to Burbine, Meibom and Binzel, publishing in *Meteoritics and Planetary Science*¹.

Astronomers once regarded asteroids as monotonously grey boulders and planetoids, the 'vermin of the skies' that interfered with their photographs of galaxies. But by the mid-1980s, astronomers had collected spectral reflectance data for hundreds of asteroids, and thousands of meteorites had been collected in Antarctica. The taxonomy of asteroids agreed with the mineralogy of meteorites: both include diverse materials ranging from iron–nickel alloy to the coal-black carbonaceous chondrites.

Some asteroids and meteorites are thought to be primitive, undifferentiated materials. But others reflect extensive chemical fractionation due to thermal heating and partial melting, yielding models of asteroidal parent bodies resembling the Earth, with a metallic core and a thick overlying olivine mantle (about three-quarters of the volume), topped with a thin basaltic crust.

Clusterings in trace-element chemistry imply that iron meteorites must come from the cores of more than 60 differentiated parent bodies. Yet there are practically no olivine-rich asteroids that might be frag-

ments of the mantles of those collisionally stripped cores — there are only seven so-called A types among about 1,000 asteroids now spectrally sampled. Similarly, of thousands of collected meteorites, only a handful (including some now thought to be martian rocks) are nearly pure olivine.

Where is the missing olivine? It is a common component of most S-type asteroids (the second most common type) and such meteorites as the undifferentiated ordinary chondrites. But from astronomers' distant perspective, it is impossible to distinguish the spatial scale of olivine, whether homogeneously mixed as fine grains, as in undifferentiated meteorites, or as huge chunks of mantle fragments mixed in a rubble pile formed when a differentiated parent body broke up and gravitationally reassembled. The latter possibility would resolve one astronomical underpinning of the olivine shortage, but it is denied by the absence of olivine-rich meteorites from such asteroids.

Meteoriticists have preferred instead to bury the missing olivine. For example, the so-called HED meteorites are basaltic, and inferred to come from the crust of a single, differentiated parent body, probably the 500-km Vesta, the only large asteroid to have the spectral signature of basalt. Indeed, despite slight colour variations revealed by recent Hubble Space Telescope images (see Fig. 1, overleaf), Vesta's thin crust remains more or less intact. Therefore, it is reasonable that Vesta's olivine-rich mantle, protected beneath its intact crust, has not been sam-



100 YEARS AGO

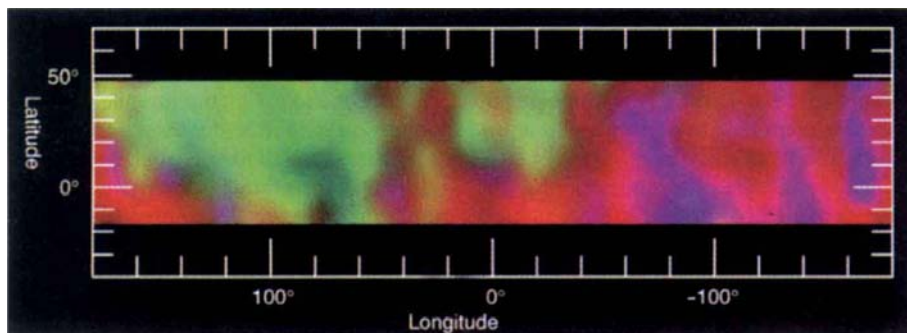
A paper on "The Monier System of Construction" was read by Mr. Walter Beer, at the Institution of Civil Engineers, on January 15. The system originated in the attempts of a Parisian florist, named Monier, to obtain large vessels of a material more durable than wood and lighter than concrete. The principle of the system is the combination of Portland-cement concrete with iron or steel in such a manner as to develop in the same material the high resistance, to compression and binding, of the former, and the great tensile strength of the latter. It has been found that in such a combination the good qualities of both materials are retained, and no chemical action occurs between the iron and the moisture in the concrete. The latter adheres firmly to the smooth surface of the metal; and the coefficients of expansion of the two constituents are for all practical purposes identical. The economy of the system in the construction of girders and arches is considerable, owing to the great strength and compactness obtained, and, further, the material is absolutely fire-proof.... The system can also be used in situations where brick and stone would be impossible.

From *Nature* 21 January 1897.

50 YEARS AGO

Mr. Winston Churchill has published two momentous articles in the *Daily Telegraph* of December 30 and 31 entitled "The Way to Stop a New War".... Let me quote his significant words.... "Of all the deterrents against war now acting upon the minds of men, nothing is comparable to this frightful agency of indiscriminate destruction [the atomic bomb]. While this supreme weapon rests in the hands of the United States alone, it is probable, though we cannot say it is certain, that a breathing space will be accorded to the world. We cannot tell how long this breathing space will last. Let us make sure that it is not cast away. If, in this interval, we can revive the life and unity of Europe and Christendom, and with this new reinforcement build high and commanding a world structure of peace which no one dare challenge, the most awful crisis of history will have passed away and the high road of the future will again become open."

From *Nature* 25 January 1947.



Vesta, goddess of the hearth, dressed up. This false-colour Hubble telescope map of the asteroid Vesta shows variations in its preserved basaltic crust.

pled. Some other meteorite types, previously thought to come from deep within the HED parent body (stony-irons and IIIAB irons), must come from some other, now disrupted, parent body or bodies — they can hardly be derived from the deep interior of still-intact Vesta!

Burbine *et al.* propose a new collisional history for the main asteroid belt that may resolve the great olivine shortage. They have invoked the latest understanding of what it takes to destroy an asteroid, including revised, lower estimates² of the fraction of projectile kinetic energy that can go into high-velocity fragments — those moving fast enough to escape from the parent body. The authors conclude that collisions among known asteroids are much too infrequent to break up the 60 or more parent bodies required to explain the variety of iron meteorites. But if the current asteroids are just the remnant of the collisional comminution of 50 times as many asteroids, then plenty of metallic cores could have been liberated by catastrophic collisions. And the mantles and crusts associated with them, made of rocks much weaker than the metallic cores, would have been 'battered to bits'. Long ago, the olivine would have been comminuted into dust and swept into the Sun.

Indeed, we see some remaining metallic cores, sandblasted clean of their once-overlying mantle rocks. An example is the 260-km asteroid Psyche, whose extreme radar reflectivity³ confirms the metallic composition tentatively inferred from reflectance spectra. In the Burbine *et al.* scenario Psyche is a lucky core, and most others have been fragmented. Even luckier are those weaker, rocky asteroids like the dark C-type asteroid Mathilde, which will be studied by the Near Earth Asteroid Rendezvous spacecraft in June. They must be like lottery winners, the rare ones that just happened to avoid any catastrophic collisions.

One would also expect some moderately lucky differentiated rubble piles to remain (not destroyed, but not wholly intact). As we get no olivine-mantle meteorites from them, they can't be common near the dynamical escape hatches from the asteroid belt through which we get meteorites, but some S-type

asteroids far from such resonances could be such differentiated rubble piles.

Vesta poses an even greater puzzle. Burbine *et al.* calculate that if the asteroids had

Behavioural ecology

Aaaaaaaaaaaaaaargh, no!

Jared M. Diamond

A friend of mine visiting India had the misfortune to join an ecotourism group whose leader foolishly followed a tiger into a thicket in order to photograph it. The outcome was fatal to the leader and emotionally devastating to the tourists, not least because of the victim's never-to-be-forgotten screams as the tiger attacked and killed him. Although it may at first seem merely natural to scream under those circumstances, a dispassionate scientist must still ask, "Why, really, did the victim put energy into screaming while struggling for his life?" This question illustrates one of the most difficult problems of behavioural ecology, the evolution of alarm signals. Douglas Chivers, Grant Brown and Jan Smith (*American Naturalist* 148, 649–659; 1996) have tackled the issue with a well-controlled laboratory experiment, substituting pike and minnows for tigers and tour guides.

Many animals give alarm signals when they detect a predator in the distance. These signals may warn the predator that it has been detect-

been more than 100 times as populous as now, there would be little chance that even one differentiated object could have survived catastrophic fragmentation and disruption. They fail to note, however, that just several times the current impact rate in the asteroid belt should have destroyed Vesta's thin basaltic crust, unless it was formed recently. Yet the HED meteorites, thought to be derived from Vesta's crust, are among the oldest known meteorites! I like much of the 'battered to bits' scenario. But then Vesta is a bigger mystery than ever.

Clark R. Chapman is in the Southwest Research Institute, 1050 Walnut Street 426, Boulder, Colorado 80302, USA.

1. Burbine, T. H., Meibom, A. & Binzel, R. P. *Meteoritics Planet. Sci.* 31, 607–620 (1996).
2. Ahrens, T. J. & Love, S. G. *Lunar Planet. Sci.* 27, 1–2 (1996).
3. Ostro, S. J., Campbell, D. & Shapiro, I. I. *Science* 229, 442–446 (1985).

ed (thereby benefiting the intended victim by dissuading the predator from attempting an attack) or may alert the signaller's kin (thereby benefiting the signaller's genes through kin selection). But it is much harder to understand the possible functions of alarm signals emitted by an item of prey, such as the tour guide, when the predator has already seized it. Alarm calls given under attack have been reported from mammals, birds, frogs and juvenile alligators. As an olfactory equivalent, specific chemicals termed alarm pheromones are released by certain snails, starfish, crustaceans, amphibians and earthworms, and many fish, when they come under attack.

This widespread phenomenon has perplexed behavioural ecologists, who have speculated — without resolution — about possible functions. Leading adaptationist hypotheses include: kin selection (as for pre-attack signals, signals given out when actually under attack may save the lives of the victim's kin by alerting them to danger); mobbing (the signals may benefit the victim

RONALD GRANT ARCHIVE



What possible functions might alarm signals such as screaming have? They may depend in part on whether, for instance, the She-Ghost of Haunted Island has her claws into her intended victim, or whether the She-Ghost has merely been sighted.

itself, by attracting conspecifics and individuals of other likely prey species that mob the predator and drive it off); and predator interference (the signals may benefit the intended victim by attracting another predator, a so-called secondary predator, which interferes with the primary predator and enables the victim to escape).

In support of this third hypothesis is the observation that predatory sharks, snakes, pike and beetles are more attracted to chemical signals from injured, disturbed or excited prey than to chemical signals from control prey. (This is the reason that human swimmers who have a bleeding wound or are holding a bleeding fish should not remain in shark-infested waters.) Nevertheless, however plausible this and other adaptationist hypotheses may appear, there has only been anecdotal evidence that secondary predators increase a prey's chance of escape, or that a prey under attack receives any other direct benefits from its alarm signals. This gap encourages a fourth, non-adaptationist, hypothesis — that the victim's scream or release of chemical is really not an alarm signal at all; rather, it serves no function and is just a by-product of the creature's attempts to escape.

To resolve this debate, Chivers *et al.* chose, as their predator and prey species, the northern pike (*Esox lucius*) and the fathead minnow (*Pimephales promelas*), which occur together in the same Canadian lakes and rivers. Both the minnows and juvenile pike live near the shore in turbid water with dense vegetation, where the pike detect invisible prey at a distance by chemical cues and then use sight to guide their close-range attack. When minnows' cells are mechanically damaged, they release an alarm pheromone that attracts pike. If a minnow survives attack, it seeks cover in the dense vegetation, remains motionless, and ceases to release alarm pheromone, thereby becoming very difficult for a pike to detect.

The authors' experimental design consisted of dividing each of 16 aquaria in half with a removable opaque barrier, putting one pike (P1, the primary predator) into one half of each aquarium, and either another pike (P2, the secondary predator; experimental tanks) or nothing (control tanks) into the other half. Two days after each pike was fed a minnow, to reinforce its interest in minnows as prey, a minnow was put into P1's side of each aquarium and the dividing barrier was removed 10 seconds after P1 had caught the minnow in its mouth. The observers then noted whether the minnow freed itself from P1, how long P1 took to consume the minnow if it did not free itself, and what (if anything) P2 did to interfere with P1's attack. Two days later, experimental conditions were reversed: the eight P1s that faced a P2 in the first trial were now given a minnow without any P2, while the eight P1s that had been allowed to catch and eat the minnow undisturbed in the first trial

were faced with a P2 in the second trial. So each individual P1 served as its own control in comparing outcomes in the presence or absence of a P2. Three P1s wimped out by not even attempting to capture the minnow, leaving 13 P1s for analysis.

The result was unequivocal. In the absence of a P2 (control tanks), no minnow escaped from the P1; all 13 P1s succeeded in swallowing their prey. In the experimental tanks, all 13 P2s interfered with P1's efforts: in ten cases, by biting P1 (especially around the gills); in three cases, by coming close to P1 and heading for the struggling minnow. In five of the 13 trials with the P2, the minnow escaped from P1, because P1 was likely to open its jaws when bitten by P2. The difference in minnow escape success between control and experimental trials was significant at the $P = 0.02$ level. For the nine P1s that consumed their minnow in both trials (in the presence and absence of a P2), the P1 took much longer to control and eat the minnow in the presence of P2 (about 290 seconds) than in its absence (a mere 104 seconds), either because P1 lost time struggling with P2 or because P1 manipulated the captured minnow more cautiously.

So interference by a secondary predator in the primary predator's attack really did increase the prey's chances of escape. Under natural conditions the difference might be magnified, because the very long 'processing' times of P1s that did succeed in swallowing their minnow despite one P2's interference may have permitted the approach of more P2s and decreased P1's chances of holding on to the minnow.

From the authors' field observations, it

seems that the same principles may well apply in the wild. The pheromone released from 1 cm² of minnow skin is detectable at a distance of about 4 m, but juvenile pike may occur spaced out at distances of less than 1 m along the shoreline, well within that distance of detection. Furthermore, even P1s undisturbed by any P2 required 104 seconds to swallow their minnow, but pike move into an area containing minnow alarm pheromone in less than 60 seconds, so that pike in the wild must often suffer interference from other pike. A pike that has just captured a minnow and ignores an intruder risks losing not just its minnow but also its life, because pike are cannibals.

This experimental confirmation of the interference arising from secondary predators may help us understand the alarm signals of any prey species, whether the signals are vocal or chemical. Chivers *et al.* quite rightly point out that the secondary-predation hypothesis is not inconsistent with other simultaneous functions of alarm signals such as kin selection: multiplicity of functions is a common feature of animal signalling. Thus, if you feel a tiger's teeth and hot breath on you, you have many reasons to scream "Aaargh, no!". You may attract other humans to come and defend you; you may entice other tigers, whose attack on the first tiger could save you at the last minute; and (even if you succumb) your last thought may be the comforting one that you warned your nearby children (who carry your genes) to escape. □

Jared M. Diamond is in the Department of Physiology, University of California Medical School, Los Angeles, California 90095-1751, USA.

AIDS

Pirated genes in Kaposi's sarcoma

Philip M. Murphy

LAST year, chemokine-receptor research unexpectedly merged with AIDS research in a flurry of landmark papers which established that HIV-1 requires certain cellular chemokine receptors for cell entry (reviewed in ref. 1). Chemokine receptors form a subgroup of G-protein-coupled receptors (GPCRs), which are 7-transmembrane-domain proteins that couple extracellular stimuli to cell responses through heterotrimeric guanine-nucleotide-binding proteins. The chemokine receptors normally couple protein ligands that are released at sites of inflammation to leukocyte chemotaxis².

On page 347 of this issue, Arvanitakis *et al.*³ add a surprising new chapter to this story by describing a virally encoded chemokine receptor — Kaposi's sarcoma-associated herpesvirus (KSHV) GPCR — which they propose may act as an oncogene in AIDS-

related malignancies, including Kaposi's sarcoma and a rare B-cell lymphoma known as primary effusion lymphoma (PEL). If they are right, KSHV GPCR could be an important target for the development of new AIDS therapies.

KSHV (human herpesvirus 8) is a γ herpesvirus that is related to the lymphocyte-transforming viruses herpesvirus Saimiri (HVS) and Epstein-Barr virus (EBV). Tight epidemiological links indicate that KSHV is an infectious cofactor for Kaposi's sarcoma and PEL^{4,5}. Its chemokine receptor, KSHV GPCR, is the product of open reading frame 74 (ORF74), which was apparently pirated from a cellular gene by the virus (see Table 1). The receptor's specificity for chemokines is not completely unexpected, because ORF74 is homologous to an HVS gene which encodes a receptor that is analogous in sequence and chemokine specificity to the

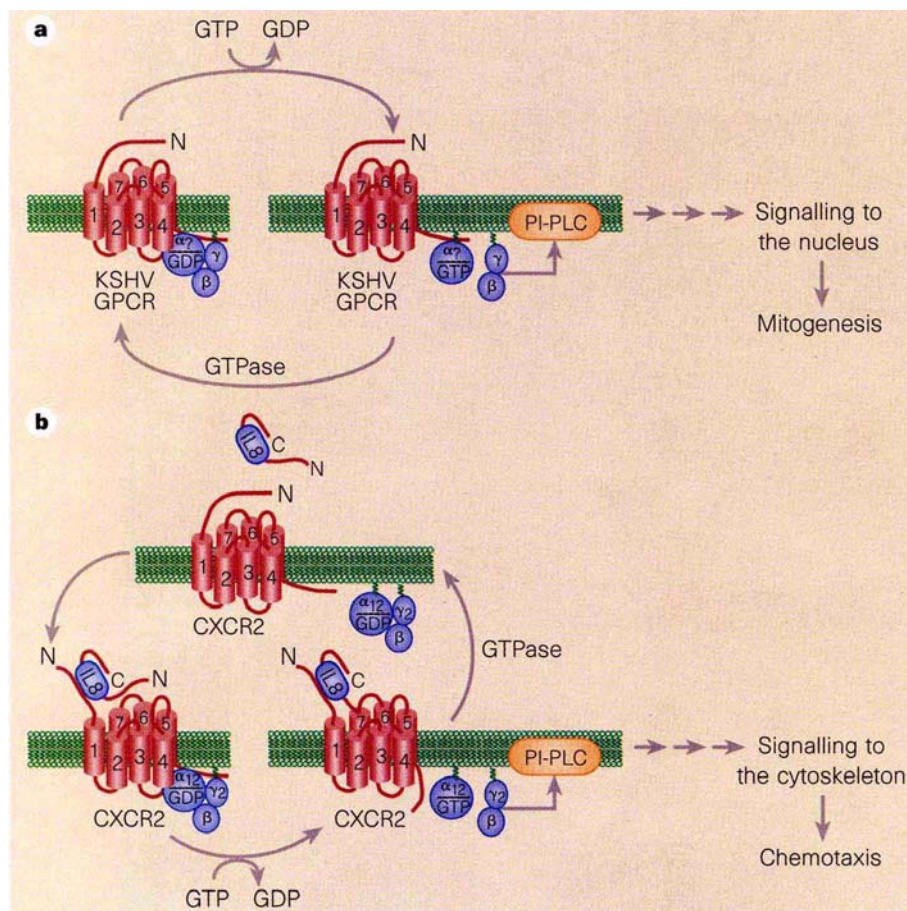


Figure 1 Model of the function of the chemokine receptor KSHV GPCR, identified by Arvanitakis *et al.*³ in virally infected cells. **a**, In the absence of chemokine ligands, KSHV GPCR activates an undefined G protein which in turn activates a mitogenic signalling pathway involving phosphoinositide-specific phospholipase C (PI-PLC). **b**, In contrast, CXCR2, the human homologue of KSHV GPCR, is active only in the presence of chemokine agonists such as IL-8. CXCR2 activates a G_q-type G protein, which initiates a chemotactic signalling pathway predominantly in non-dividing cells such as neutrophils. In each case, the receptor promotes guanine nucleotide exchange (GTP for GDP) at the α-subunit of the heterotrimeric G protein. Nucleotide exchange causes dissociation of the GTP-bound α-subunit from the β/γ subunits, which then activate downstream effectors. Activation is terminated by the intrinsic GTPase activity of the α-subunit, which restores the α-subunit to the GDP-bound form. The α-subunit then reassociates with the β/γ subunits, completing the G-protein cycle.

human chemokine receptor CXCR2 (ref. 6).

What is unexpected is that KSHV GPCR is fully active in the absence of chemokine ligands. It is able to activate the phosphoinositide pathway in COS-1 cells, and *in vitro* transfection of rat fibroblasts with ORF74 leads to cell proliferation. Remarkably, its constitutive (agonist-independent) activity is comparable to maximal agonist-dependent activity for CXCR2, which does not signal constitutively. Moreover, chemokine ligands do not affect its activity — either positively or negatively — and, although direct G-protein coupling has not been shown, KSHV GPCR does not seem to use the types of G proteins that are used by other known chemokine receptors.

The chemokine superfamily has over 30 members, most of which can be classified into one of two groups, CXC and CC, distinguished by the presence (CXC) or absence (CC) of a single amino acid between the first two of four conserved cysteines. CXCR2 is specific for CXC chemokines, whereas KSHV GPCR binds both CC and CXC chemokines, including two (I-309 and platelet factor-4) whose cellular receptors have not been identified. Of the 11 known human chemokine receptors, only one, the Duffy antigen, binds both classes of chemokines. But in primary sequence, Duffy is less closely related to KSHV GPCR (20 per cent identity) than is CXCR2 (25 per cent identity).

Of the chemokines tested, interleukin (IL)-8 binds to KSHV GPCR with the highest affinity (K_d , 30 nM). IL-8 is angiogenic, and mitogenic for endothelial cells⁷; however, the IL-8 mitogenic signalling pathway is not defined, and cellular chemokine receptors have not been carefully tested for mitogenic activity. Perhaps KSHV uses KSHV GPCR to connect constitutively to the pathway that is used for IL-8-induced angiogenesis — this could explain the intense angiogenesis that is present in Kaposi's sarcoma.

Nothing is known about the function of native KSHV GPCR protein in either Kaposi's sarcoma or PEL cells, despite the fact that KSHV GPCR messenger RNA has been detected in both cell types⁸. So far, the KSHV genomes that have been studied are

from PEL rather than Kaposi's sarcoma cells⁹. If there are genetic differences between Kaposi's sarcoma and PEL strains, there could also be differences in the function of KSHV GPCR in the two diseases, possibly contributing to the marked differences in their biology. Moreover, many cases of PEL (but not Kaposi's sarcoma) harbour both EBV and KSHV, and it will be important to test whether signalling by KSHV GPCR may be modulated by EBV-encoded proteins.

It will not be easy to progress beyond the circumstantial link between KSHV GPCR and KSHV-associated pathogenesis. Not only has it been difficult to model the biology of Kaposi's sarcoma *in vitro* or in animals, but the KSHV life cycle is not defined and, although the virus has been cultured *in vitro*,

Table 1 Viral homologues of chemokines and chemokine receptors

ORF*	Virus	Cellular homologue	Specificity†
K4	KSHV	CC chemokine	CCR5
K6	KSHV	CC chemokine	?
K2	Molluscum contagiosum	CC chemokine	?
ECRF3	Herpesvirus Saimiri	CXCR2	CXC chemokines
US28	Human cytomegalovirus	CCR1	CC chemokines
74	KSHV	CXCR2	CC and CXC chemokines
74	Equine herpesvirus-2	CXCR2	?
E1	Equine herpesvirus-2	CCR3	?
K2R	Swine pox virus	CXCR2	?
Q2/3L	Capripox virus	CCR4	?

* Abbreviations: ORF, Open reading frame; KSHV, Kaposi's sarcoma-associated herpesvirus;

CXCR, CXC chemokine receptor; CCR, CC chemokine receptor.

† Known human ligand or receptor for the indicated viral gene product.

systems for creating targeted mutations in KSHV have not yet been developed. KSHV lacks homologues of transforming genes such as those possessed by HVS and EBV, and ECRF3, the HVS homologue of KSHV GPCR, is distinct from the known HVS oncogene¹⁰: the function of ECRF3 in HVS biology has not been defined.

Kaposi's sarcoma behaves more like hyperplasia (uncontrolled growth of normal cells) than an autonomous neoplasm (abnormal clonal growth of tissue), whereas the opposite is true for PEL. Sustained immunodeficiency favours growth of Kaposi's sarcoma *in vivo*, and its characteristic spindle-shaped cells, considered to be the 'tumour' cells, grow poorly *in vitro*. Growth is improved when cytokines such as IL-1, IL-6 and oncostatin M or the HIV-1 Tat protein are added, leading to suggestions of a 'growth-factor theory' of pathogenesis¹¹. In this regard, the genomic sequence of KSHV, which was reported last month⁹, has much to offer beyond KSHV GPCR: homologues of the cytokine IL-6, and the cell-cycle-control proteins bcl-2 and cyclin D, are present. Viral cyclin D mimics human cyclin D in preventing retinoblastoma-mediated cell-cycle arrest^{12,13}, and viral IL-6 mimics human IL-6 in preventing mouse myeloma-cell apoptosis¹³. So the KSHV sequence merges infectious and growth-factor theories to form a unified theory of Kaposi's sarcoma pathogenesis, where the relevant growth factors may (in part) be oncogenes that are encoded by the causative infectious agent.

Whether there is a master transforming oncogene (as there are for acutely transforming retroviruses and HVS), and whether all of the pirated genes contribute to proliferation of the infected cell, remains to be seen. As Arvanitakis *et al.*³ point out, the mitogenic mechanism for KSHV GPCR in particular could be indirect (see Fig. 1 on page 297), involving the induction of cellular factors such as basic fibroblast growth factor, which is associated with the development of Kaposi's sarcoma.

KSHV GPCR will interest pharmacologists because it is a rare, naturally occurring illustration of a step in the allosteric ternary-complex model of receptor activation. This was first proposed by Lefkowitz to interpret the behaviour of GPCRs that become constitutively active when they are mutated experimentally¹⁴. In the model, the receptor exists in a 'constrained' or inactive conformation, in which productive receptor-G-protein contact is unlikely. Agonists promote a brief, reversible allosteric transition of cytosolic domains of the receptor from the constrained conformation to a 'relaxed' or active conformation in which contact is favoured. A single amino-acid change in the receptor can irreversibly mimic the effect of agonists, and allow sustained signalling.

Some other wild-type GPCRs have low

levels of constitutive activity, and the naturally occurring mutant thyroid-stimulating hormone and luteinizing hormone-chorionic gonadotropin receptors have high constitutive activity resulting from somatic point mutations. These mutations form the genetic basis for the thyroid and pituitary neoplasms in which they were found, resonating with the theory that KSHV GPCR may drive KSHV-associated cell proliferation¹⁵.

It is still possible that a 'preferred' chemokine agonist for KSHV GPCR exists, or that an antagonist could be made for potential therapeutic application. IL-8 binds 30 times more weakly to KSHV GPCR than to CXCR2; however, many known chemokines have not been tested and new ones are constantly being discovered. Interestingly, KSHV itself encodes two pirated CC chemokine candidates — vMIP-I and vMIP-II. Chang and colleagues reported last month¹³ that vMIP-I inhibits HIV-1 cell entry mediated by the chemokine receptor CCR5. So, could viral MIP-I regulate the spread of HIV-1 in Kaposi's sarcoma tumours? And is the interaction of vMIP-I with CCR5 incidental or essential to the KSHV life cycle, or does it act in a virally

encoded autocrine loop with KSHV GPCR, expropriated from the host? Whatever the answer, the significance of KSHV GPCR lies in its potential to lead to new treatments for patients with Kaposi's sarcoma, and to stimulate the search for novel functions for cellular chemokine receptors. □

Philip M. Murphy is in the Laboratory of Host Defenses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 10, Room 11N113, Bethesda, Maryland 20892, USA.

1. Fauci, A. S. *Nature* **384**, 529–534 (1996).
2. Murphy, P. M. *Cytokine Growth Fact. Rev.* **7**, 47–62 (1996).
3. Arvanitakis, L., Geras-Raaka, E., Varma, A., Gershengorn, M. C. & Cesarman, E. *Nature* **385**, 347–350 (1997).
4. Chang, Y. *et al. Science* **265**, 1865–1869 (1994).
5. Weiss, R. A. *Nature Med.* **2**, 277–278 (1996).
6. Ahuja, S. K. & Murphy, P. M. *J. Biol. Chem.* **268**, 20691–20694 (1993).
7. Koch, A. E. *et al. Science* **258**, 1798–1801 (1992).
8. Cesarman, E. *et al. J. Virol.* **70**, 8218–8223 (1996).
9. Russo, J. J. *et al. Proc. Natl Acad. Sci. USA* **93**, 14862–14867 (1996).
10. Murthy, S. C. S., Trimble, J. J. & Desrosiers, R. C. *J. Virol.* **63**, 3307–3314 (1989).
11. Ensoli, B., Barillari, G. & Gallo, R. C. *Immunol. Rev.* **127**, 147–155 (1992).
12. Chang, Y. *et al. Nature* **382**, 410–412 (1996).
13. Moore, P. S., Boshoff, C., Weiss, R. A. & Chang, Y. *Science* **274**, 1739–1744 (1996).
14. Lefkowitz, R. J., Cotecchia, S., Samama, P. & Costa, T. *Trends Pharmacol. Sci.* **14**, 303–307 (1993).
15. Sande, J. V. *et al. J. Clin. Endocrinol. Metab.* **80**, 2577–2585 (1995).

Mycology

The business of fructification

Roy Watling

In certain parts of the world, fungi are a prized food — for example, there is a long history of collecting and eating wild mushrooms in southeast Asia and in Europe, especially in the Nordic and Slavic cultures. In Britain, by contrast, the consumption of exotic mushrooms is relatively new, although there have always been a few avid collectors. Demand is now high, however, to the extent that good sites in southeast England are attracting pickers who sell their bounty to local restaurants and foreign markets, a practice that has spawned much press coverage.

New markets are opening up all the time, and in consequence the already considerable numbers of popular species consumed are likely to rise. For instance, the world production of chanterelles (*Cantharellus* spp.), all from the wild, ranges from 150,000 to 200,000 metric tonnes a year, in a season that runs from May to December. The value in the market place is about £1 billion (\$1.67 billion).

So mushrooms can be big business. It is for that reason, and because of the underlying scientific issues, that Eric Danell and

Mushrooms in the market place — *Boletus* and chanterelles on sale in the rue de Seine, Paris.

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Francisco J. Comacho's item of Scientific Correspondence in this issue (*Nature* **385**, 303; 1997) will attract attention. The authors describe how they have made the first breakthrough in growing the golden chanterelle (*C. cibarius*) in artificial culture.

These days, supermarkets stock a whole range of exotic mushrooms — the penny bun (*Boletus edulis*), various species of chanterelle, the horn of plenty (*Craterellus* spp.) and hedgehog fungus (*Hydnum repandum*), for instance — as well as the familiar button mushroom (*Agaricus hortensis*) and the wood-rotting oyster and black forest mushrooms (*Pleurotus* spp. and *Lentinula edodes*). The difference between these old 'reliables' and the new interlopers is that the latter, including the chanterelle, are ectomycorrhizal.

Worldwide, over 85 per cent of vascular plants form an intimate relationship with a fungus or fungi, which enhances their growth. In most trees — the pines, oaks and beeches of the Old and New World forests, dipterocarps of southeast Asia, eucalypts of Australia and caesalpinoid legumes of West Africa — the fungus is found as an envelope of tissue surrounding the root-tips with a network of hyphae running between the outer cells but not penetrating them. Such relationships are called sheathing or ectomycorrhizas. The hyphae constitute the vegetative stage of the life cycle, and for the greater part of that cycle ectomycorrhizal fungi are members of the underground soil ecosystem. It is only in the sexual stage, when they produce the brightly coloured, often edible (but sometimes poisonous) fruiting bodies that much attention is paid to them — and then sadly by few scientists.

As Danell and Comacho point out, it is more than a century since Frank published the first comprehensive observations on ectomycorrhizas (*Ber. Deutsch. Bot. Ges.* **3**, 128–145; 1885). Most work since Frank has been disappointing, in that fruiting of the fungal partner in pure culture, when it happens, is often by chance and is not repeatable. Alas, we know very little about the induction of fruiting-body primordia in these ectomycorrhizal fungi, a necessary step if the fungus is to be domesticated.

Following others, Danell and Comacho inoculated a host tree under artificial conditions using a pure culture of a chanterelle. This was originally isolated by Danell several months before from a Swedish fruit-body. What they (and no others) have now achieved is that, in the experiments, fungal sheaths were formed that gave rise to new fruiting bodies.

The big question will be whether these results can be repeated consistently. At the moment, out-planting of trees inoculated with the fungus can be envisaged, but even then the persistence of the inoculum at the

root surface will be unpredictable. The new technique could have conservation applications, but the big prize — both scientific and economic — will be understanding the link between fungus and host tree. Only then can suitable factors be supplied to sustain the fungal partner without the need to grow the trees.

In the supermarket, of course, the fruiting body is isolated from the total community — the soil community, that is. In being a member of the soil ecosystem the chanterelle is exposed to all the dynamism, heterogeneity and so on that this implies. Details of these interactions constitute one of the great remaining mysteries in mycology. What is essential in any further work is to probe and thereby come to an appreciation of the relationships between fungus, tree and soil microorganisms. The life cycle of some ecto-

mycorrhizal fungi is probably much more complex than we realize, with tripartite (or more) relationships being involved.

The fact that chanterelles can now be fructified in culture, even if only with their host, gives hope to those wishing to go further and understand why, in certain respects, the chanterelle is different from many other ectomycorrhizal species. For instance, in Britain in 1995, every common ectomycorrhizal fungus had caught up and overcome the unseasonably dry spring and summer by the end of the year — except the chanterelle. Did the primordia weather the winter, and the May collections of 1996 represent those initially intended for 1995? At the least, we now have an experimental procedure for tackling such questions. □

Roy Watling is at the Royal Botanic Garden, 20A Inverleith Row, Edinburgh EH3 5LR, UK.

Plasma physics

A commotion over turbulence

Benjamin A. Carreras

Achieving sustained fusion by magnetic confinement is a complex business. Its complexity sometimes leads to paradoxical situations. In the past year, the biggest scientific achievement in fusion research has been the effective elimination of turbulent transport in the plasma core; this has led to a leap in performance in the leading tokamak experiments^{1–3}. Yet concerns have now been raised in the media⁴ that turbulent transport will cripple the International Thermonuclear Experimental Reactor (ITER), the largest fusion experiment currently being designed. How can we understand this apparent contradiction?

To produce energy from fusion reactions, the fuel (usually deuterium and tritium) has to be at a temperature of around 10^8 K. At these temperatures, matter exists as a plasma, with electrons stripped from their nuclei. It has to be confined well enough for the energy loss rate to be less than the fusion power. So energy transport in confined plasmas is a critical issue.

Confinement by magnetic fields relies on the fact that charged particles stream along the magnetic field lines, following a helical trajectory. They can also diffuse perpendicular to field lines by Coulomb collisions, but that is a relatively slow process — usually too slow to explain observed heat loss. In most cases, plasma turbulence is the main cause of the cross-field transport.

During the 1980s, experiments showed that the energy transport induced by plasma turbulence increases with heating power. In this 'low-confinement mode', the confinement time decreases as one over the square root of the power input. That situation was not promising. But in 1982 a new mode of

operation was discovered⁵, the first of the advanced confinement modes that were to follow. The result has been a steady improvement in the DIII-D tokamak; for example, the performance (density times ion temperature over confinement time) has doubled every two years since the mid-1980s. The new experiments^{1–3}, in which turbulent transport is effectively suppressed and confinement times increased by a factor of five or six, are the most recent results of this process.

One of the underlying mechanisms in the transition to a higher-confinement regime is the creation of transport barriers by flow shear (see box). The effective increase in the overall plasma confinement depends on the location and the radial width of the barrier. The best results so far improve confinement over two-thirds of the plasma radius. This mechanism is universal — it works whatever the precise nature of the turbulence⁶.

It is no surprise that the plasma turbulence problem is not fully solved. Even in classical fluids, turbulence isn't well understood, and in magnetically confined plasmas the problem is much more complex, involving a number of charged turbulent fluids (electrons and the different ions), coupled through an electromagnetic field. But despite the intrinsic difficulties, a picture of the mechanism for ion turbulence in the plasma core of tokamaks was emerging by the mid-1980s.

The main cause of turbulence is thought to be a linear instability in the ion temperature gradient⁷. Based on analytical approximations, turbulent diffusivities have been derived that provide essential insight into the turbulence dynamics, although their range of practical validity is limited by those approximations.

One significant development of the numerical techniques is the assumption that the diffusivity is a simple function of the mixing length⁸ which can be determined by fast, linear calculations that give the dominant parameter dependences times a coefficient that is a slow function (determined through numerical turbulence calculations) of dimensionless parameters of the reactor set-up⁹. This has led to the IFS/PPPL model, named after the main institutions involved in its development. The basis for this approach has yet to be validated through detailed high-resolution turbulence calculations, and it is important that details of such calculations be published soon.

One reason we are interested in these models is to predict the performance of future devices, like ITER. To have a predictive energy-transport model requires many other components besides the turbulence-induced diffusivity. Dorland and Kotschenreuther have combined the IFS/PPPL diffusivity model with an assumed prescription for the density evolution and the boundary temperatures, and proposed it as a predictive transport model. Their results imply that ITER will fall far short of its main goal — a self-sustaining fusion reaction⁴. But other transport models, some theory-based and some empirical, have also been used to predict ITER's performance. Some are optimistic and some pessimistic; all are being tested against the existing experimental database to assess their reliability¹⁰. So far, the Dorland and Kotschenreuther model has not produced the best agreement or the worst.

ITER was initially planned to operate in the low-confinement mode, but all the models showed that it wouldn't then be able to achieve its goals. The regime now under consideration is the first of the advanced operation modes, the one discovered in 1982. But studying a single operation mode gives a very narrow perspective for such a device. The more advanced high-confinement regimes, with spontaneous or actively driven internal transport barriers, should be considered. They are not yet proven to be compatible with the steady state, but experiments underway may do so over the next few years.

ITER, being a large engineering project dealing with frontier physics, must be designed with enough flexibility to accommodate new physical developments. In recent years, fusion science has progressed quickly, so we must retain the ability to incorporate the advances that fusion science is making throughout the design and construction phases of ITER. □

Benjamin A. Carreras is at the Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, USA.

1. Lazarus, E. A. *et al.* *Phys. Rev. Lett.* **77**, 2714–2717 (1996).
2. Levinton, F. M. *et al.* *Phys. Rev. Lett.* **75**, 4417–4420 (1995).
3. Koide, K. *et al.* *Phys. Plasmas* (in the press).
4. Glanz, J. *Science* **274**, 1600–1602 (1996).
5. Wagner, F. *et al.* *Phys. Rev. Lett.* **49**, 1408–1412 (1982).
6. Burrell, K. *Phys. Plasmas* (in the press).
7. Coppi, B., Rosenbluth, M. N. & Sagdeev, R. Z. *Phys. Fluids* **10**, 582–587 (1967).
8. Kadomtsev, B. B. *Plasma Turbulence* (Academic, New York, 1965).
9. Kotschenreuther, M. *et al.* *Phys. Plasmas* **2**, 2381–2389 (1995).
10. Connor, J. W. *et al.* in *IAEA Fusion Energy Conference 1996* (IAEA, in the press).
11. Charlton, L. A. *et al.* *Phys. Plasmas* **1**, 2700–2710 (1994).
12. Biglari, H., Diamond, P. H. & Terry, P. W. *Phys. Fluids B* **2**, 1–4 (1990).

Daedalus

The dark is light enough

All life, the textbooks tell us, depends on the Sun. At the base of the food chain, green plants use sunlight to convert carbon dioxide into plant tissues. All higher organisms, directly or indirectly, live on what the green plants have won.

Recently, however, a genuine non-solar ecology has been discovered. The oceanic 'black smokers', those abyssal eruptions of geothermally superheated water, are far too deep for sunlight to penetrate. Yet they support a bizarre and complex ecology of specialized bacteria, tube worms and crustacea. These creatures are supposed to get their energy by oxidizing metal sulphides dissolved in the hot water.

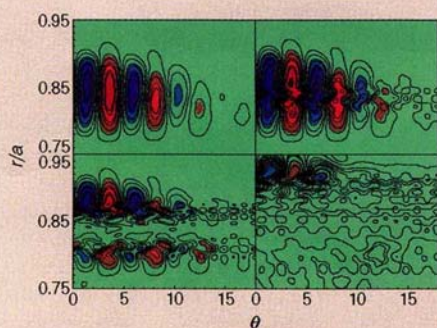
Daedalus has other ideas. Our own motors and power stations exploit the classic thermodynamic source of useful energy, temperature differences. A black smoker creates a temperature difference approaching 400 degrees, exploitable at 60% efficiency (compared to about 10% for photosynthesis). Surely, says Daedalus, specialized organisms must have evolved to use this rich source of energy. A tube worm, for example, can be over a metre long. With one hot and one cold end, it could operate neatly as a thermal engine. But even tiny bacteria could gain energy by eddying in and out of the hot zone, thus taking themselves repeatedly round a thermal cycle.

These 'Carnot creatures' would operate by chemistry rather than physics. The high temperature would initiate a cascade of productive chemical reactions, whose waste heat would be discharged at the low temperature. No biochemist would recognize such a weird metabolism unless he was looking for it; but the search should be well worth while. For Carnot creatures could fit usefully into human industry. Many motors and furnaces throw out waste heat at hundreds of degrees. A suitable 'Carnot culture' of organisms could exploit that heat usefully, especially if their unique biochemistry could be adapted to cleaning up related metallic or sulphide pollution.

But Carnot biochemistry could be far more important than this. Many worlds, from distant 'brown dwarf' stars to the satellites of giant planets, may have internal heating but no effective 'Sun'. If Carnot life is possible, it may well have evolved in such dark and dismal places — making life abundant throughout the Universe. Indeed, our distant descendants may be able to harness Carnot biochemistry to sustain themselves on geothermal or residual brown-dwarf warmth when the Sun finally grows dim.

David Jones

Shear calm



Plasma turbulence and transport are highly nonlinear. As a consequence, many states exist for a single set of boundary conditions, and spontaneous transitions can occur from one solution to another. The low- and high-confinement regimes are

examples of two such states.

At low levels, injected power goes into heating the plasma and increasing the temperature gradient, and so increasing the turbulence level. But the turbulence increases energy transport, reducing the gradient and the

confinement time — so the heating efficiency decreases with increasing power. This state is disordered and turbulent.

At higher power, above a threshold value, injected power can mostly go to heating the plasma, and, through a process of self-organization, building up a radial electric field. That induces a global $E \times B/B^2$ flow, which carries turbulent vortices along with it. A radial gradient in the field causes the flow to shear, which tends to shear the vortices and smooth out density fluctuations (the figure shows a simulated set of turbulent eddies being distorted and broken up¹¹). This reduces fluctuation-induced transport¹², so confinement time is increased. B.A.C.

Yulii Khariton (1904–96)

Physicist, instrumental in developing Soviet nuclear weapons

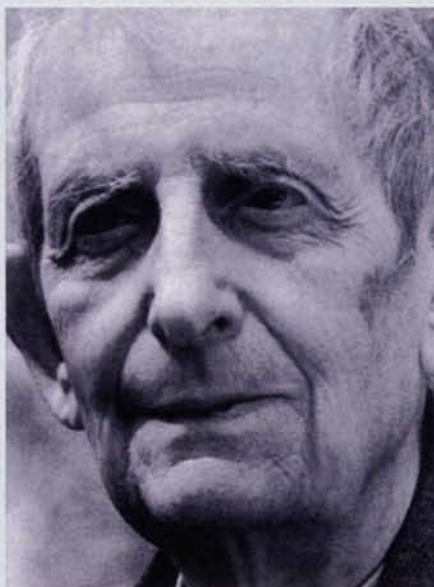
Yulii Borisovich Khariton, who died on 19 December last year at the age of 92, was a key figure in the Soviet nuclear weapons programme. For over 40 years he was scientific director of Arzamas-16, the Soviet equivalent of Los Alamos.

Khariton was born into a literary and artistic family in St Petersburg in 1904, and throughout his life retained the manners and interests of a Russian intellectual. He studied physics at the Polytechnical Institute, and was invited by Nikolai Semenov (who received the Nobel prize for chemistry in 1956 for his work on chemical chain reactions) to do research at the Leningrad Physicotechnical Institute, the leading Soviet centre of physics at the time. In 1926 he went to Cambridge, where he spent two years at the Cavendish Laboratory, working under Ernest Rutherford and James Chadwick on the sensitivity of the eye to weak impulses of light, and on alpha radiation. He received his PhD in 1928.

Many years later, Khariton commented on the coincidence that he and J. Robert Oppenheimer — his counterpart in the US nuclear weapons programme — had been born in the same year, had the same first name (Julius), and had mothers who instilled in them a love of music, art and poetry. Oppenheimer spent a short time at the Cavendish in 1926, but he and Khariton did not meet there.

On his way back to the Soviet Union Khariton visited Germany, where his mother was living. He decided, after seeing the dangerous political situation there, to do defence-related research. On his return to Leningrad he created a laboratory to study explosives, which soon became part of Semenov's new Institute of Chemical Physics.

After the discovery of nuclear fission at the end of 1938, Khariton's thoughts turned to the possibility of nuclear chain reactions. With Yakov Zeldovich he produced a series of classic papers in 1939–41 on the conditions under which a chain reaction would take place in uranium. The two physicists investigated both slow-neutron and fast-neutron reactions, and calculated that 10 kg of uranium-235 would be sufficient for an atomic bomb. Soviet nuclear research stopped when Hitler invaded the Soviet Union in June 1941. At the end of 1942, however, Stalin decided to



initiate a small Soviet project after receiving intelligence about British and American work on the atomic bomb. Igor Kurchatov, a friend and colleague of Khariton's since the 1920s, was made scientific director. Khariton took charge of the work on the bomb, but continued with his work on conventional munitions at the same time. Khariton was a member of the Soviet atomic mission sent to Germany in May 1945. The Soviet Union had little to learn from Germany about the bomb, and most of the leading German nuclear scientists had fled to the West. But Khariton and a colleague did track down over 100 tonnes of uranium oxide — an invaluable find for the Soviet project, which was very short of uranium.

After Hiroshima, Stalin turned the Soviet project into a crash programme. Khariton chose a site at Sarov, 400 kilometres east of Moscow, and assembled a group of very capable scientists and engineers to work on the bomb. Zeldovich was the first head of the theoretical department, and Andrei Sakharov worked there from 1950 until 1968. This heavily guarded, ultra-secret installation, known as Arzamas-16, was the only nuclear weapons institute until a second one was established in the Urals in 1955.

The first Soviet fission bomb was detonated successfully on 29 August 1949 at a special test site in Kazakhstan. This was a copy of the first US plutonium bomb; when it became public knowledge in the early 1990s that the Soviet Union had been able to use intelligence information to re-create the US design, Khariton defended that decision as the quickest way to build a Soviet bomb. A more effective Soviet

design was detonated in 1951. The first Soviet hydrogen bomb was tested in August 1953, and the first two-stage thermonuclear weapon in November 1955.

Khariton was in many ways a surprising choice as chief designer of nuclear weapons. His two years in the West made him politically suspect. So, too, did the fact that his parents lived abroad: his mother emigrated to Palestine from Germany in the 1930s; and his father, who lived in Riga before the war, was arrested and shot when the Red Army occupied the Baltic states in 1940. Yet Khariton remained untouched even by the anti-semitic campaign of the late 1940s. He met Stalin only once, but he had to work closely with Lavrentii Beria, the head of the secret police, whom he found efficient and correct in his dealings with scientists.

Khariton remained scientific director of Arzamas-16 until 1992. His approach to design and development was careful and thorough: "We have to know ten times more than we are doing" was his motto. He saw it as his duty to ensure that the Soviet leaders received sound technical advice on nuclear weapons issues.

Until the 1980s Khariton remained a very secret figure. When I met him in 1988, I was the first Westerner he had encountered since the Second World War. He was a quiet and courteous man, with a probing intelligence, similar in manner and outlook to other European physicists of his generation. In conversation he was unassuming about his own role in history. He gave the impression of being a man of great intellectual integrity, but one absorbed in his work rather than engaged in broad political issues. He was in this respect very different from Sakharov, whom he greatly admired.

Khariton was showered with honours by the Soviet government, but became a public figure at a time when the state he had served was disintegrating and the weapons he had helped to create were no longer needed on anything like the same scale. Yet he did not yearn for the past, and was conscious of the human and environmental costs of the nuclear weapons programme. When he was invited in 1995 to give the Oppenheimer memorial lecture at Los Alamos, he very much wanted to accept the invitation, but ill health prevented him from doing so. In the text of the lecture he sent to Los Alamos he expressed the hope that "those who come after us will find the way, will find in themselves the firmness of spirit and the decisiveness not to do harm in striving to do good". □

David Holloway

David Holloway is in the Center for International Security and Arms Control, Stanford University, 320 Galvez Street, Stanford, California 94305, USA.

Successful cultivation of the golden chanterelle

The golden chanterelle (*Cantharellus cibarius*) and allied species are highly appreciated edible mushrooms. Large quantities of *Cantharellus* species are exported¹ from the northwestern coast of the United States to central Europe, where the *C. cibarius* population is declining². There have been many efforts to cultivate this species^{3–5}, and here we report the first successful fruit-body formation in the greenhouse, hosted by pine seedlings only 16 months old.

Cultivation of the golden chanterelle has been hampered by the difficulty of obtaining pure mycelia, owing to contamination by other organisms in the fruit-body, especially *Pseudomonas* bacteria⁶. Also, unlike cultivated saprobic mushrooms that can utilize cellulose, *C. cibarius* obtains its carbohydrates from the ectomycorrhizal symbiosis it forms with trees. Common techniques for establishing mycorrhiza using fast-growing plant nursery fungi are not applicable to *Cantharellus*, so a routine method for *C. cibarius* mycorrhiza formation was not published until 1994 (ref. 7). However, by that time it was known from observations in nature that *C. cibarius* in Sweden was mostly found with trees older than 25 years.

In January 1995 we started an *in vitro* mycorrhiza synthesis procedure, using Swedish *C. cibarius* mycelia that we had isolated from fruit-body tissue in 1988. In March 1995, we transferred *Pinus* seedlings inoculated with *C. cibarius* into pots, from which we harvested plants during August 1995 to study mycorrhizal establishment. We returned a fraction of the plants to their

pots and left them in the greenhouse for another seven months.

In April 1996 we found the first fruit-body, which we harvested later that month. It originated from a drain-hole in a plastic pot carrying *Pinus sylvestris* inoculated with Swedish *C. cibarius*. The size of the fruit-body was 3.5 cm, and its odour, trama and spore-forming hymenium were normal (see Fig. 1). We found fluorescent *Pseudomonas* in great quantities as in wild strains of *C. cibarius*. The fruit-body was not attached to roots or mycelial cords. Another pot contained a 0.5-cm fruit-body primordium and several contained large quantities of hyphal knots resembling young primordia. We found a third large fruit-body in June 1996. In November 1996 there was a second flush of three more fruit-bodies.

We found no obvious factors triggering fruit-body formation. The *Pinus* seedlings were 16 months old, demonstrating that *C. cibarius* is not dependent on old trees for reproduction. In addition, the fungal isolates used in this experiment had been in culture for eight years. It has been 112 years since Frank published the first observations on mycorrhiza⁸, and only now can we control the life cycle of a mushroom that is an important symbiont to forest trees as well as a highly appreciated food item.

With greenhouse production, it would be possible to study, for example, the physiology of reproduction, genetics, and bacterial and insect symbiosis. Obviously, the life-cycle strategy of *C. cibarius* is different from inedible mycorrhizal model organ-



Figure 1 First cultivated fruit-body of the golden chanterelle (*C. cibarius*).

isms such as *Laccaria*, which quickly colonizes roots via spores. The *Cantharellus* cultivation technique could be applied to other edible or endangered species such as *Tricholoma matsutake*. Its life cycle is similar to *Cantharellus*, and it is the most valuable mushroom in the world (US\$100 per fruit-body). There are also potential commercial applications similar to the establishment of truffle (*Tuber melanosporum*) orchards by outplanting of inoculated seedlings⁹.

Eric Danell

Department of Forest Mycology and Pathology,
Swedish University of Agricultural Sciences,
PO Box 7026, 750 07 Uppsala, Sweden
e-mail: Eric.Danell@mykopat.slu.se

Francisco J. Camacho

Department of Botany & Plant Pathology,
Oregon State University,
Corvallis, Oregon 97331-29024, USA

- Schlosser, W. E. & Blatner, K. A. *J. For.* **93**, 31–36 (1995).
- Arnolds, E. *Can. J. Bot.* **73**, (suppl. 1), S987–S998 (1995).
- Danell, E. thesis, Univ. Uppsala (1994).
- Straatsma, G. et al. *Trans. Br. Mycol. Soc.* **85**, 689–697 (1985).
- Fries, N. *Mycologia* **71**, 216–219 (1979).
- Danell, E., Alström, S. & Ternström, A. *Mycol. Res.* **97**, 1148–1152 (1993).
- Danell, E. *Mycorrhiza* **5**, 89–97 (1994).
- Frank, B. *Ber. Deutsch. Bot. Ges.* **3**, 128–145 (1885).
- Hall, I., Brown, G. & Byars, J. *The Black Truffle* (NZ Inst. Crop Food Res., Christchurch, 1994).

Thalidomide's chirality

In her News article¹, Rubner wrote that “pharmaceutical products need to be tested separately for the effects of left- and right-handed molecules, following the tragedy in the early 1960s when women gave birth to children with malformed limbs after being given the sedative thalidomide, which had been contaminated with the wrong enantiomer”. This statement about thalidomide relies on a report² demonstrating malformations in pups of rodents treated with S-thalidomide, whereas offspring of animals treated with the R-form developed normally. We point out here, however, that these data need to be interpreted with caution.

First, rodents are generally insensitive to the teratogenic effects of thalidomide³. Second, Fabro et al.⁴ demonstrated that there is

no difference in teratogenicity between the two enantiomers of thalidomide in New Zealand white rabbits, a species susceptible to the teratogenic effect of thalidomide. Third, and most important from our point of view, Eriksson et al.⁵ have shown that there is a rapid interconversion between the two forms of thalidomide *in vitro* and *in vivo* in humans after application of either S- or R-thalidomide. The mean rate constants for *in vivo* inversion are between 0.12 and 0.17 h⁻¹, respectively, depending on whether the S-form or the R-form is applied. Therefore, about 8 h after application of the pure enantiomers, an equilibrium has been reached and both forms of the compound are present in the blood at similar concentrations. This leads to the conclusion that application of a pure enantiomer would not have prevented the tragedy of thalidomide-induced embryopathy.

Thalidomide has attracted new interest because of its immunomodulating activity⁶, which is reflected in *in vitro* assays by an inhibition of the release of tumour necrosis factor (TNF)-α from activated mononuclear human blood cells. To find out whether this inhibitory effect is enantio-selective, we tested a set of configuration-stable thalidomide analogues as well as the enantiomers of thalidomide *in vitro*⁷. Surprisingly, we found a clear enantio-selectivity towards the S-form in all the analogues tested.

In the case of thalidomide, the S-form confers a slight, but statistically significant, increase in the degree of TNF-α inhibition compared with the R-form. It should be taken into account that the racemization is not complete during the first, most sensitive hours of this assay. Enantio-selectivity towards the S-form has also been observed with configuration-stable — or at least

slower-racemizing — thalidomide derivatives in sedation⁸ and teratogenicity⁹, which suggests that desired and adverse effects cannot be separated by stereochemistry.

In summary, two lines of evidence contradict the hypothesis that use of a pure enantiomer as a drug substance would have prevented the tragedy of thalidomide: the rapid racemization of thalidomide enantiomers; and the lack of evidence for a stereospecific separation of sedation and immunomodulation from teratogenicity.

Stephan Wnendt*

Kai Zwingenberger†

*Department of Molecular Pharmacology,
Research and Development, and

†Medical Scientific Division,

Grünenthal GmbH, 52078 Aachen, Germany

1. Rubner, J. *Nature* **382**, 104 (1996).

2. Blaschke, G., Kraft, H. P., Fickentscher, K. & Kohler, F. *Drug Res.* **29**, 1640–1642 (1979).

3. Scott, W. J., Fradkin, R. & Wilson, J. G. *Teratology* **16**, 333–336 (1977).

4. Fabro, S., Smith, R. L. & Williams, R. T. *Nature* **215**, 296 (1967).

5. Eriksson, T., Björkman, S., Roth, B., Fyge, A. & Höglund, P. *Chirality* **7**, 44–52 (1995).

6. Zwingenberger, K. & Wnendt, S. I. *Inflammation* **46**, 177–211 (1996).

7. Wnendt, S. et al. *Chirality* **8**, 390–396 (1996).

8. Büch, H. P., Omior, G. & Knabe, J. *Drug Res.* **40**, 32–36 (1990).

9. Heger, W. et al. *Teratog. Carcinog. Mutagen.* **14**, 115–122 (1994).

Energy saving in huddling penguins

The emperor penguin (*Aptenodytes forsteri*) is the only animal to breed during the severe Antarctic winter. Because they feed exclusively at sea, breeding on sea-ice means that the males, which assume the entire task of incubation, fast for 105–115 days¹. Here we describe how these animals conserve enough energy to survive the harsh Antarctic conditions while incubating eggs.

We determined the rate of body-fuel loss by calculating changes in body mass and body water. We found that the behaviour of huddling in groups enables males to

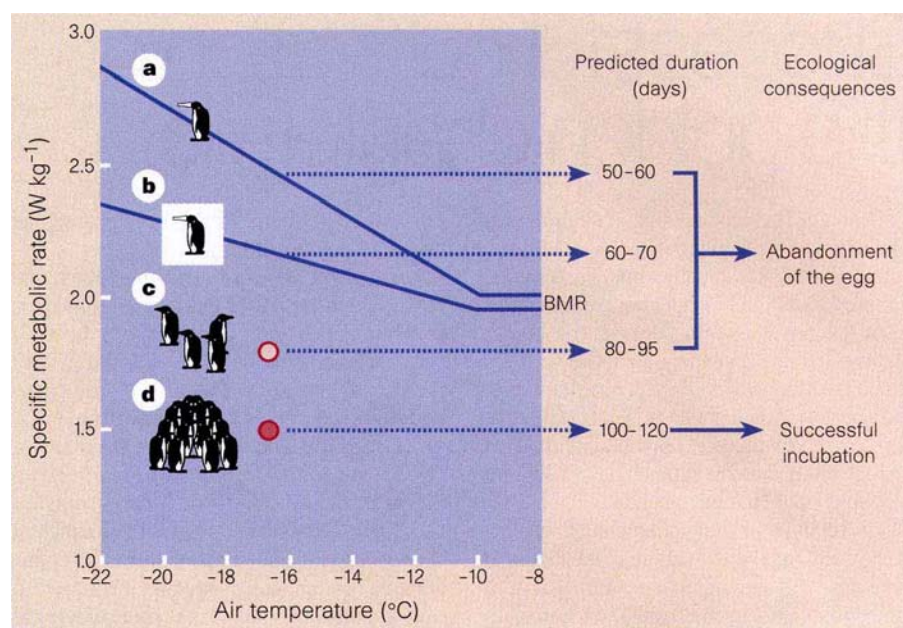


Figure 1 Specific metabolic rate (metabolic rate per unit body mass) of emperor penguins as a function of air temperature in four situations: individual bird wearing a mask for measurement of metabolic rate from gas exchange, either (a) under natural conditions² or (b) in a controlled temperature chamber³; birds whose metabolic rate has been calculated from changes in body composition (present study), for either (c) birds retained captive outdoors in small groups, without the ability to huddle efficiently, or (d) birds free-ranging in their colony. Further details of the methods can be obtained from Y. L. M. Predicted duration of fasting is calculated from the specific metabolic rate and from previous data on body-mass loss and body composition^{1,2,9}. Incubation success is possible only for birds that huddle. BMR, basal metabolic rate.

maintain a metabolic rate 25% below that measured in an individual bird at thermoneutrality^{2,3}. Males that are isolated, and therefore unable to huddle, cannot cope with the long fast necessary for successful incubation.

We studied penguins in the Pointe Géologie colony (66.7° S, 140.0° E) in Adélie Land. At the beginning of the winter (April), when mating occurs and birds have already fasted for about a month, we weighed, blood-sampled, and injected 36 males with a known amount of deuterated water, to allow us to monitor body-water loss.

Of these males, we kept two groups of

five birds in fenced, 15 m² outdoor areas. Conditions were similar to those in the colony (mean air temperature, -16.4 °C; mean wind speed 10.7 m s⁻¹; 38 days of snowfall). Penguins in small groups are unable to huddle efficiently⁴. This therefore enabled a comparison with the remaining 26 deuterium-enriched birds, which were released into the colony of about 3,000 birds. The captive birds were weighed every 5 days and blood sampled every 15 days. In addition, five of them were re-injected monthly to determine the pattern of decrease of total body water.

To avoid breeding failure, we deter-

Table 1 Changes in body composition during the winter fast

	Fast duration (days)	Initial (kg)	Final (kg)	Daily loss (g day ⁻¹)	Initial (kg)	Final (kg)	Daily loss (g day ⁻¹)	Water efflux (g day ⁻¹)	Water influx (g day ⁻¹)	Metabolic water (g day ⁻¹)	Water intake (g day ⁻¹)
Free-ranging	96 (10)	37.2 (2.2)	23.9 (0.8)	137 (13)	17.3 (1.0)	14.4 (1.2)	29.7 (10.4)	151.6 (19.3)	121.9 (13.2)	109.5 (20.2)	12.4 (25.8)
Captive	97 (16)	38.6 (1.9)	22.2 (0.6)	171 (27)	18.2 (1.1)	14.3 (1.2)	45.0 (15.9)	206.3 (35.4)	160.9 (31.8)	144.5 (19.6)	16.5 (27.6)
Free – captive (%)			+71	-19.9			-34.0	-26.5	-24.2	-24.2	
		Lipid mass			Protein mass			Metabolic rate		Specific metabolic rate	
		Initial (kg)	Final (kg)	Lipid use (g day ⁻¹)	Initial (kg)	Final (kg)	Protein use (g day ⁻¹)	(W)		(W kg ⁻¹)	
Free-ranging		11.8 (1.6)	2.2 (1.5)	98.4 (19.9)	7.7 (0.3)	6.9 (0.4)	8.4 (2.9)	46.5 (8.6)		1.5 (0.3)	
Captive		12.0 (0.9)	0.8 (1.6)	116.0 (18.5)	8.0 (0.3)	6.8 (0.3)	12.7 (4.5)	55.4 (8.5)		1.8 (0.3)	
Free – captive (%)											
					-4.1		-33.9	-16.1		-16.5	

Values are means (with s.d.) for male emperor penguins, free or captive. The data from the two groups of captive birds were pooled because they did not differ significantly. The differences (free – captive) between the eight free-ranging and 10 captive males are presented only when they are significant (Student's *t*-test, $P < 0.05$). Total body water was calculated from deuterium dilution using a previously described equation relating lipid mass to body mass¹⁰ (which was corrected to account for the mass of skeleton and feathers). Total body water is overestimated by 1.1% by this method. As the decrease in total body water is linear with time, we used equations 4 and 6 of ref. 11 to calculate water fluxes. Metabolic water production was calculated assuming that water production through lipid oxidation is 1.07 ml water g⁻¹ and 0.50 ml water g⁻¹ is through protein oxidation¹². Lipid mass = body mass – 5.465 – ((total body water – 1.938)/0.77); protein mass = 0.944 × (body mass – lipid mass – total body water). To calculate metabolic rate, we used the following coefficients¹²: energy derived from 1 g lipid, 39.31 kJ; energy derived from 1 g protein, 17.81 kJ.



Figure 2 Male emperor penguins huddling together in midwinter.

mined the body-water content of the 26 free-ranging labelled birds only at the end of incubation, during the few hours when the female returns to take over the egg or newly hatched chick and before the male leaves to feed at sea. We were able to recapture 8 of the 26 marked males.

Although the birds had the same initial body mass and endured a fast of the same duration as the captive birds, the final body mass of the free-ranging penguins was 7% larger than for captive birds. Water flux rates were 25% lower in free-ranging than in captive birds and their daily water loss was 34% lower. Even so, both groups of birds had the same low water intake, of about 15 g per day. Emperor penguins at the Pointe Géologie colony have little access to water during their winter fast, except on warmer days when snow falls. Eating snow represented only 11% of the metabolic water production through the oxidation of body fuels (see Table 1).

Free-ranging birds had metabolic rates ~16% lower than captives (Table 1). This difference cannot be attributed to activity levels, as emperors are almost motionless during the winter¹. Because both fenced and free-ranging birds were exposed to similar climate conditions, the lower metabolic rate in free-ranging birds can be partly attributed to the energy saving resulting from huddling. Without huddling, emperor penguins would reach the metabolic status that would trigger refeeding⁵ three weeks earlier. The egg would then be abandoned before the return of the female (see Fig. 1).

The metabolic rate of fenced and free-ranging penguins was respectively 8% and 25% below the basal metabolic rate previously measured from respiratory gas exchange^{2,3} (Fig. 1). How can the metabolic rate of emperors under natural conditions be lower than the basal metabolic rate measured in an individual bird, and the difference be even greater in huddling birds? It could be partly due to a sleep-induced energy saving. The proportion of sleep increases during the long winter fast of emperor penguins⁶ and sleep lowers the metabolic rate by 8% in the little penguin, *Eudyptula minor*⁷.

Huddling in emperor penguins (Fig. 2) is possible because of the absence of territorial

behaviour in this species, which is unique among penguins. As the presumed ancestors of emperor penguins lived at the temperate latitude of New Zealand⁸, huddling can be considered as the key adaptation for energy saving that has enabled the birds to breed in one of the coldest environments on Earth.

André Ancel*, Henk Visser†

Yves Handrich*, Dirkjan Masman††

Yvon Le Maho*

*Centre d'Ecologie et Physiologie Énergétiques,
Centre National de la Recherche Scientifique,
23 rue Becquerel,

67087 Strasbourg Cedex 02, France

†Zoological Laboratory, University of Groningen,
PO Box 14, 9750 AA Haren, The Netherlands

and Centre for Isotope Research,

University of Groningen, Nijenborgh 4,

9747 AG Groningen, The Netherlands

‡Institute for Veterinarian Research,

PO Box 81, 8090 AB Wezep, The Netherlands

e-mail: yvon.lemaho@c-strasbourg.fr

1. Prévost, J. *Ecologie du Manchot Empereur* (Expéditions Polaires Françaises, Hermann, Paris, 1961).
2. Le Maho, Y., Delclitte, P. & Chatonnet, J. *Am. J. Physiol.* **231**, 913–922 (1976).
3. Pinshow, B., Fedak, M. A., Battles, D. R. & Schmidt-Nielsen, K. *Am. J. Physiol.* **231**, 903–912 (1976).
4. Jouventin, P. in *The Biology of Penguins* (ed. Stonehouse, B.) 435–446 (Macmillan, London, 1975).
5. Le Maho, Y., Robin, J.-P. & Cherel, Y. *News Physiol. Sci.* **3**, 21–24 (1988).
6. Dewasmes, G., Buchet, C., Geloen, A. & Le Maho, Y. *Am. J. Physiol.* **256**, R476–R480 (1989).
7. Stahl, C. D., Megirian, D. & Nicol, S. C. *J. Comp. Physiol. B* **154**, 487–494 (1984).
8. Simpson, G. G. *Penguins. Past and Present, Here and There* (Yale Univ. Press, New Haven, 1976).
9. Robin, J.-P., Frain, M., Sardet, C., Groscolas, R. & Le Maho, Y. *Am. J. Physiol.* **254**, R61–R68 (1988).
10. Groscolas, R. *Comp. Biochem. Physiol. A* **90**, 361–366 (1988).
11. Nagy, K. A. & Costa, D. P. *Am. J. Physiol.* **238**, R454–R465 (1980).
12. Schmidt-Nielsen, K. *Animal Physiology: Adaptation and Environment* 4th edn (Cambridge Univ. Press, Cambridge, 1990).

Fugu genome is not a good mammalian model

The Japanese pufferfish (*Fugu rubripes*) has a very compact genome, about eight times smaller than that of humans, but with a similar gene repertoire, and has been proposed as a model for vertebrate gene analysis¹. It has also been suggested that the *Fugu* genome could be used as a tool for the identification of disease genes, provided that significant regions of similarity exist between *Fugu* and mammalian genomes^{2–4}. Few studies have addressed the extent of similarity^{2,4,5}. The only examples of significant conservation of gene order over short regions are in specialized loci such as the *Hox* loci, where genomic organization is believed to be critically related to developmental regulation^{2,5}. We have studied the unique spatial arrangement and clustering of the mammalian *Surfeit* genes that constitute the tightest mammalian gene cluster

described so far, and report here that the organization of these genes in the *Fugu* and mammalian genomes differs greatly.

The well-characterized mouse *Surfeit* locus (Fig. 1a) is composed of at least six housekeeping genes, whose organization and juxtaposition is conserved in humans^{6,7} (T. Duhig *et al.*, manuscript in preparation) and chicken⁸, but completely different in the two invertebrates studied, the fruitfly (*Drosophila melanogaster*) and a nematode (*Caenorhabditis elegans*)^{9,10}. The human *Surfeit* locus is located at chromosome band 9q34.1 (ref. 7) and the mouse locus is located in an equivalent region on the proximal portion of mouse chromosome 2 (ref. 11).

Fugu homologues of six *Surfeit* genes have been isolated by screening a gridded *Fugu* genomic cosmid library (G. Elgar and D. Nizetic, unpublished data) with mammalian *Surfeit* gene complementary DNA probes at reduced stringency. We isolated positive cosmids and sequenced the DNA regions in and around the *Fugu Surfeit* homologues. These homologues are all highly conserved at the amino-acid level and their gene structures are mostly identical to the mammalian genes (N. A., J. G. and M. F., manuscript in preparation). Investigation of their genomic environments reveals that the homologues are found at three loci in the *Fugu* genome (Fig. 1b).

The *Fugu Surf-1*, *Surf-3/rpL7a* and *Surf-6* gene homologues are clustered together, the *Surf-2* and *Surf-4* homologues are adjacent and the *Surf-5* homologue is found alone. However, even when the homologues are found together at the same locus, their gene order and genomic organization are mainly different to those found in mammals.

The *Fugu Surf-3* and *Surf-1* homologues are the only genes that show the same relative genomic organization as in mammals (Fig. 1). The *Surf-6* gene has a completely novel position with respect to the *Surf-1/Surf-3* gene pair when compared with mammals. The mammalian *Surf-6* gene is 3' to the *Surf-3* gene, with the *Surf-5* gene in between (Fig. 1a), whereas the *Fugu Surf-6* gene is found immediately 5' to the *Surf-1* homologue (Fig. 1b), a position occupied by the *Surf-2* gene in mammals. At a separate locus, the *Fugu Surf-2* and *Surf-4* homologues are adjacent; however the two transcription units are in series in the *Fugu* genome, whereas in mammals they are convergent (Fig. 1).

Strong sequence homology to the coding region of the mammalian argininosuccinate synthetase (ASS) gene is found 3' to the *Fugu Surf-4* homologue and DNA sequences 5' to the *Fugu Surf-2* gene show strong homology to human-expressed sequence tag EST00098. These findings are of particular interest because the human ASS gene, like the human *Surfeit* genes, is located at chromosome band 9q34.1 but is positioned

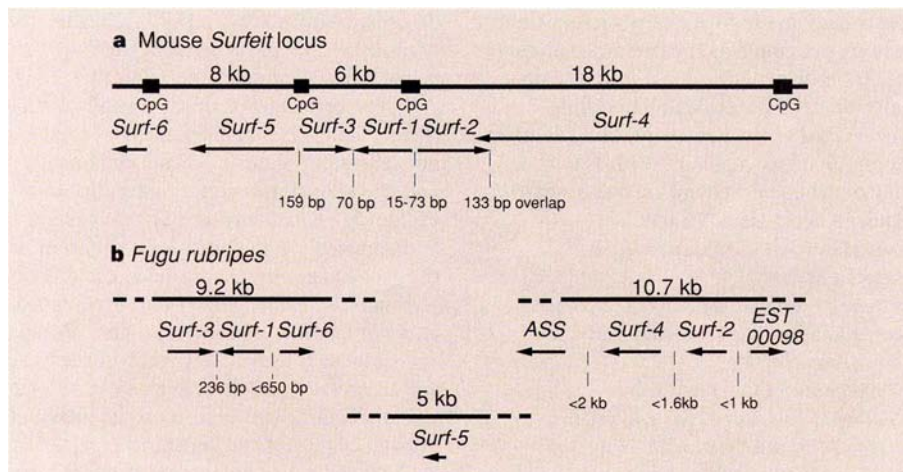


Figure 1 Comparison of genomic organization of the *Surfeit* genes in mouse and *Fugu*. **a**, The mouse *Surfeit* locus (modified from ref. 16). Continuous line represents genomic DNA; distance between the genes is shown and the direction of transcription is indicated by arrows. The 5' end of each gene is associated with a CpG island (boxes). Organization and juxtaposition of the *Surfeit* genes is conserved in humans^{6,7}, and the human *Surfeit* locus is located at chromosome band 9q34.1 (ref. 7). **b**, Organization of the *Surfeit* gene homologues, ASS homologue and conserved EST00098 in *Fugu* shown as three separate loci based on cosmid contigs and DNA sequencing. The sequenced genomic regions are represented by continuous lines with the number of kilobases sequenced and the distances between the coding regions of the genes indicated. The direction of transcription of each of the homologues is shown by arrows.

about 2 megabases from the *Surfeit* locus¹², and EST00098 has also been mapped to human chromosome 9 (ref. 13). Our polymerase chain reaction analysis shows that EST00098 is not located within 40 kilobases of either side of the human *Surfeit* locus.

The *Fugu Surf-5* gene is the only *Surfeit* gene homologue found at a third separate locus. Sequences upstream of *Fugu Surf-5* and *Surf-3* and downstream of *Surf-5* and *Surf-6* are not significantly similar to any sequences in the GenBank database.

Comparative Southern-blot analysis of *Fugu* genomic DNA and cosmid DNA reveals that the cloned DNAs analysed are not rearranged with respect to their true genomic organization, and that the *Fugu* gene homologues all have a single copy in the genome. We have accurately positioned the *Fugu* homologues within each cosmid relative to vector arms, using detailed restriction-enzyme mapping and Southern-blot analyses, and find that none of the unrelated cosmid overlaps. Preliminary construction of cosmid contigs around each of the three *Fugu Surf* gene loci demonstrates that they must be separated by between 60 and 100 kilobases in the *Fugu* genome, if not located at completely different chromosomal locations. The inability to link the cosmids in a contig suggests that the three clusters are relatively isolated, despite the condensed nature of the *Fugu* genome.

Our results show that the genomic organization of the six *Surfeit* genes, the ASS gene and EST00098 are different in *Fugu* and mammals. Our observations suggest that local DNA rearrangements, such as small inversions, have been common in one or both lineages that gave rise to *Fugu* and

mammals. They also show that comparative gene order is so often different between *Fugu* and mammals that it cannot be assumed with confidence that genes that are linked in *Fugu* will also be linked in mammalian genomes. The human *Surfeit* locus genes are located within the candidate region for tuberous sclerosis 1 disease gene⁷ and the ASS gene is linked to the loci for nail-patella syndrome¹⁴ and idiopathic torsion dystonia¹⁵. Our observations bring into question the use of the compact *Fugu* genome as a tool to accelerate the identification of these or other human disease genes using comparative mapping or positional cloning strategies.

Jonathan Gilley, Niall Armes

Mike Fried*

Imperial Cancer Research Fund, PO Box 123,
Lincoln's Inn Fields, London WC2A 3PX, UK
e-mail: fried@europa.lif.icnet.uk

1. Brenner, S. *et al.* *Nature* **366**, 265–268 (1993).
2. Elgar, G. *et al.* *Trends Genet.* **12**, 145–150 (1996).
3. Little, P. *Nature* **366**, 204–205 (1993).
4. Trower, M. K. *et al.* *Proc. Natl Acad. Sci. USA* **93**, 1366–1369 (1996).
5. Aparicio, S. *et al.* *Proc. Natl Acad. Sci. USA* **92**, 1684–1688 (1995).
6. Williams, T., Yon, J., Huxley, C. & Fried, M. *Proc. Natl Acad. Sci. USA* **85**, 3527–3530 (1988).
7. Yon, J., Jones, T., Garson, K., Sheer, D. & Fried, M. *Hum. Mol. Genet.* **2**, 237–240 (1993).
8. Colombo, P., Yon, J., Garson, K. & Fried, M. *Proc. Natl Acad. Sci. USA* **89**, 6358–6362 (1992).
9. Armes, N. & Fried, M. *Mol. Cell. Biol.* **15**, 2367–2373 (1995).
10. Armes, N. & Fried, M. *Mol. Cell. Biol.* **16**, 5591–5596 (1996).
11. Stubbs, L. *et al.* *Genomics* **6**, 645–650 (1990).
12. Henske, E. P. & Kwiatkowski, D. J. *Genomics* **28**, 105–108 (1995).
13. Polymeropoulos, M. H. *et al.* *Nature Genet.* **4**, 381–386 (1993).
14. Henske, E. P., Ozelius, L., Anderson, M. A. & Kwiatkowski, D. J. *Genomics* **13**, 841–844 (1992).
15. Ozelius, L. J. *et al.* *Am. J. Hum. Genet.* **50**, 619–628 (1992).
16. Garson, K., Duhig, T., Armes, N., Colombo, P. & Fried, M. *Genomics* **30**, 163–170 (1995).

*To whom correspondence should be addressed.

Emplacement of Taupo ignimbrite

Dade and Huppert¹ present a model for emplacement of the Taupo ignimbrite (New Zealand) from a turbulent dilute pyroclastic current (0.3% solids by volume). This model contrasts sharply with that of a concentrated current (tens of per cent solids by volume) previously proposed by me² to explain this remarkable deposit. Differences between the two models are important for how ignimbrites are interpreted and in reconstructing their causative eruptions, and centre around two key contrasting views of the Taupo pyroclastic current.

The first is that the model in ref. 1 has the current driven by the momentum of the overall dilute system, whereas my model implicitly has the current driven primarily by momentum of the concentrated basal portion. In my model, an overlying dilute cloud is considered to have still been present but to have represented the product, not the driver, of the concentrated basal portion. Second, in ref. 1, turbulence in the current is *required* to transport particles, whereas in my model, turbulence is only one of several possible particle support mechanisms during transport, including grain–grain interaction, buoyancy effects, matrix support and fluidization³. The key distinction between the models is thus in evidence for the value and distribution of solids concentration in the current, not the presence or otherwise of turbulence. In this regard, three aspects of ref. 1 are not soundly based when measured against the data that I presented in ref. 2.

First, the apparent match between modelled and measured parameters in the ignimbrite presented in Fig. 2a–c of ref. 1 is not real. Dade and Huppert use my estimate of the whole-ignimbrite grain-size distribution (Fig. 57 of ref. 2) as the initial input for their model, but match their predictions to data from only one portion of the ignimbrite, the layer-2 veneer facies, previously emphasized^{2,4} to be unrepresentative. Compared with the whole ignimbrite, the veneer is depleted in coarse lithics (found in layer 1) and pumices (found in layer-2 ponded facies). To test Dade and Huppert's theory against observations requires measurements of lateral variations in the deposit as a whole. On the other hand, vertical and lateral grain-size variations seen in the Taupo ignimbrite are consistent with deposition from a concentrated current with varying degrees of fluidization and turbulence².

Second, Dade and Huppert's model fails to account for four key features of the ignimbrite, considered critical in establishing my model. One of these is the presence of

the fines-rich, pumiceous layer 1(P), overlain by the fines-poor lithic-rich layer 1(H), then by the fines-rich, pumiceous layer-2 deposits, all emplaced with a strong lateral velocity component. The ignimbrite thus cannot simply be the result of particle fallout from a turbulent dilute suspension as these three layers, individually or collectively, show no systematic vertical ordering by any grain-size or inferred aerodynamic characteristic applicable in gaseous suspensions.

Another feature of my model is that field and grain-size evidence indicates that many fluidization-segregation bodies in the ignimbrite formed within a moving concentrated current (for example, sedimenting to form the widespread, regionally distributed layer 1(H); ref. 5) and not simply after the flow had come to rest. Observations² and experimental data^{6,7} imply that these segregation bodies can only form within concentrated systems.

Furthermore, there are marked changes in the fines and lithic contents of ponded layer-2 deposits at 55–60 km from the vent. These changes are qualitatively consistent with the model adopted for a concentrated stratified flow², but cannot be explained by interaction of a dilute current with the topography (as suggested in ref. 1), as the changes occur along flat as well as mountainous flow paths.

Finally, field and grain-size evidence in the ignimbrite argues for a more dilute and highly turbulent current in proximal areas (≤ 13 –20 km from the vent)², for example, abundant megaripples in the layer-2 veneer deposits, lack of systematic grading in layer-2 valley ponds, scarcity of layer 1(P) deposits, and so on. The contrast between these proximal features and those seen elsewhere in the Taupo ignimbrite (which match those seen in numerous other 'conventional' ignimbrites³) supports the concept of a concentrated transport system for most of the travel distance of the current.

Third, the claimed matching of pumice:lithic size ratios in the ignimbrite with theory (Fig. 1 of ref. 1) is false. With the reasonable estimates of pumice and lithic densities given by Dade and Huppert, the W_b/W_l ratio in the ignimbrite (both as a whole and in individual facies) is not uniform at about 1.0 as they imply, but varies from <0.2 in proximal areas (≤ 20 km), through 0.5 to 3 in medial (20–55 km) areas to 0.5–1 in distal areas (> 55 km), based on my data used in ref. 2 (here W_b/W_l is the ratio of the settling velocity of the largest pumice fragments to that of the lithic fragments). Pumice and lithic sizes (all lithics measured for ref. 2 were demonstrably vent-derived) in the ignimbrite do not match aerodynamically for a dilute suspension.

In summary, the field and grain-size evidence available from the Taupo ignimbrite rule out the model in ref. 1, and imply that

the Taupo flow was concentrated for most of its travel distance. A chief weakness of Dade and Huppert's model is that any exponential variations of parameters with distance from the vent could equally well be modelled by the radial spreading of a concentrated, axisymmetrical current. The principles are similar (preferential or partial sedimentation from poorly sorted material in an axisymmetrical, radially moving transport medium), it is merely that the rates may differ.

C. J. N. Wilson

*Institute of Geological and Nuclear Sciences,
Private Bag 2000,
Taupo 2730, New Zealand*

Dade and Huppert reply—The debate on the mechanism of ignimbrite emplacement has been frustrated by lack of quantitative, predictive models for the parent flows. We¹ took Wilson's careful observations on the Taupo deposit² and reconstructed the parent flow consistent with the deposit and the assumptions of a model for a dilute, deposit-forming gravity current. The definition of dilute is set by the experimentally established range of applicability of quantitative models for such flows. Using our model, we calculated a near-vent solids concentration of 0.3% by volume, but model trends in deposit characteristics are largely independent of the precise value of this estimate.

The key message of our paper¹ was that our model can be used to predict regional variations in the geometry and sorting of grain sizes in the bulk of the Taupo deposit. Quantitative prediction of the dramatic trends in deposit architecture seen at Taupo, on the other hand, has not yet been demonstrated for a concentrated parent flow. Accordingly, one should carefully reconsider the qualitative interpretation of the Taupo deposit given by Wilson², particularly before applying it to this and other ignimbrites of low aspect ratio. We acknowledged in our paper that a more complicated quantitative model is required to explain all the details of the Taupo deposit. In our view, finding fault with some of the details of our paper (which, because of its simplicity, is easy to do) is not useful in the absence of a testable alternative.

In this regard we challenge Wilson to pursue his case. Although it is true that the Taupo ponded facies (layer 2b) is locally enriched in coarse pumice relative to the veneer facies, these layer-2 deposits are otherwise indistinguishable in bulk composition and grain size. Under these circumstances it is not clear what weight should be given to local features in a unit (layer 2b) which, at least morphologically, reflects local depositional conditions. We focused on the landscape-mantling veneer facies because it is broadly representative of

the regional conditions that controlled the deposition of layer 2. Layer 2 makes up 82% of the total volume (30.5 km³) of the Taupo ignimbrite, so we used Wilson's estimate of the initial overall grain-size distribution as an input to the model. The good agreement between the calculated and observed trends shown in Fig. 2 of our paper requires no further justification. For the sake of complete reporting, we presented the lateral trends observed in layer 1, and discussed possible explanations for the differences between layers 1 and 2. We pointed out that the sedimentological differences between layer 1 and the veneer deposit are much greater than the differences between the veneer and layer 2b.

It is unclear whether the general description of lateral trends in sedimentological properties of the Taupo deposit by a quantitative model which provides for abrupt changes at 55–60 km can be justified in a statistically significant way. To the extent to which discontinuities can be resolved, we pointed out that they are not unique to any specific flow regime and could be the result of the effects of stratification in a turbulent parent flow. We also suggested that abrupt sedimentological changes could record the interaction of the parent flow with complex regional topography which, at these distances, comprises ridge-like obstacles that are up to 500–1,000 m above vent level. Application of our model does not require these events, but we note that the lateral extent of hydraulic influence of topography is proportional to its horizontal dimension⁸, which can be up to several tens of kilometres in the Taupo region. One could expect to observe secondary and complicated topographic effects not only in layer 2b of the locally controlled ponded facies (where most of the potential discontinuities are seen) but throughout the entire deposit.

Our calculations of W_b/W_l (Fig. 1 of ref. 1) demonstrate the approximate settling equivalence expected for particles deposited from a dilute flow. The calculations are based on our equation 2, which is valid for all particle-settling Reynolds numbers, and the material properties given in our paper. The lateral distributions of the maximal sizes of lithics are given by expressions in the captions of Figs 9 and 37 of ref. 2. The lateral distribution of maximal size of pumice in layer 2 is given by a 'hand-fitted' envelope to the data in Fig. 39 of ref. 2 and generated in a manner consistent with that used by Wilson for the distribution of lithics. Without doubt, reasonable differences in the values taken for material properties, descriptions of lateral trends in maximal sizes and expression for particle settling speed can result in small differences in W_b/W_l . Wilson's efforts appear to be a case in point. Although Wilson does not give their basis, his calculations demon-

strate the approximate settling equivalence of large pumice and lithics in that W_b/W_l is generally near unity. As we noted in our paper, low values of the ratio near the vent probably reflect the scarcity of large pumice in the initial suspension.

W. Brian Dade

Herbert E. Huppert

*Institute of Theoretical Geophysics,
Department of Earth Sciences,
and Department of Applied Mathematics &
Theoretical Physics,
University of Cambridge,
Cambridge CB2 3EQ, UK*

1. Dade, W. B. & Huppert, H. E. *Nature* **381**, 509–512 (1996).
2. Wilson, C. J. N. *Phil. Trans. R. Soc. Lond. A* **314**, 229–310 (1985).
3. Sparks, R. S. J. *Sedimentology* **23**, 147–188 (1976).
4. Walker, G. P. L., Wilson, C. J. N. & Froggatt, P. C. *J. Volcanol. Geotherm. Res.* **9**, 409–421 (1981).
5. Walker, G. P. L., Self, S. & Froggatt, P. C. *J. Volcanol. Geotherm. Res.* **10**, 1–11 (1981).
6. Wilson, C. J. N. *J. Volcanol. Geotherm. Res.* **20**, 55–84 (1984).
7. Druitt, T. H. *J. Volcanol. Geotherm. Res.* **65**, 27–39 (1985).
8. Baines, P. G. *Topographic Effects in Stratified Flows* (Cambridge Univ. Press, 1995).

Sound alters visual motion perception

Little is known about how complementary inputs from different senses are coordinated. To explore the perceptual consequences of this coordination, we devised simple visual stimuli whose analogues outside the laboratory ordinarily produce distinctive sounds. Our assay, optimized with visual stimuli whose motion could be seen in either of two ways¹, reveals that sound can alter the visual perception of motion.

A computer displayed two identical objects that moved steadily towards one another, coincided, and then moved apart. This display is consistent with two different interpretations: either, after coincidence, the two objects could have continued in their original directions; or they could have collided and then bounced, reversing directions. Collisions often produce sounds characteristic of the materials and force of the impact². Our experiments determined whether introduction of sounds would promote the perception of bouncing.

The targets, small disks, moved in three different ways. In two conditions the disks paused at the point of their coincidence, for either one frame or two. In another condition the disks moved continuously with no interruption. These visual conditions were presented together with a brief click (2.5 ms; sound pressure level 75 dB) either 150 ms before or after coincidence, or at the point of coincidence. A control condition presented no sound. Each stimulus combination was presented 20 times in random order to ten naive observers. After each trial, the observer reported whether the disks appeared to stream through or bounce off each other.

Sound at or near the point of coincidence promotes perception of 'bouncing' compared with the control condition (see Fig. 1; repeated measures analysis of variance, $P < 0.0001$). A sound just before visual coincidence has nearly as much effect as a sound at coincidence, but a sound after coincidence has significantly less effect ($P < 0.01$), although it still enhances the perception of bouncing. There is consider-

able tolerance for asynchrony between sound and visual inputs: even when the sound is delayed by 150 ms after coincidence, the likelihood of seeing bouncing increases ($P < 0.05$). As others have reported^{3,4}, the overall proportion of bouncing responses grows with increasing pause duration ($P < 0.001$).

The effect of sound on visual motion could represent some generalized attention effect, evocable by any salient transient. We examined this possibility by testing three conditions with 15 new observers. In one condition, a sound (440 Hz, 100 ms, 80 dB) came on only at the point of coincidence. In another condition the same sound, equally intense, was on for the entire visual display, but was turned off for 100 ms at the moment of coincidence. In the final condition no sound was presented. As before, sound onset significantly increased bouncing reports ($P < 0.01$). However, sound offset produced results indistinguishable from those with no sound at all. This suggests that the sound's impact on visual motion is not the product of heightened attention at the moment of coincidence, but may require an acoustic event that signals a collision between moving objects.

The origin of the effect of sound on visual motion is unknown; it may involve some form of multisensory cells⁵ and/or feedback from higher-level, multisensory areas onto primary motion areas⁶. Research combining psychophysics and brain imaging may reveal the nature of this effect, and of audiovisual interactions more generally.

Robert Sekuler

*Volen Center for Complex Systems,
Brandeis University,
Waltham, Massachusetts 02254, USA
e-mail: sekuler@volen.ccs.brandeis.edu*

Allison B. Sekuler

*Department of Psychology,
University of Toronto, Toronto,
Ontario M5S 3G3, Canada*

Renee Lau

*Dana Hall School,
Wellesley,
Massachusetts 02181, USA*

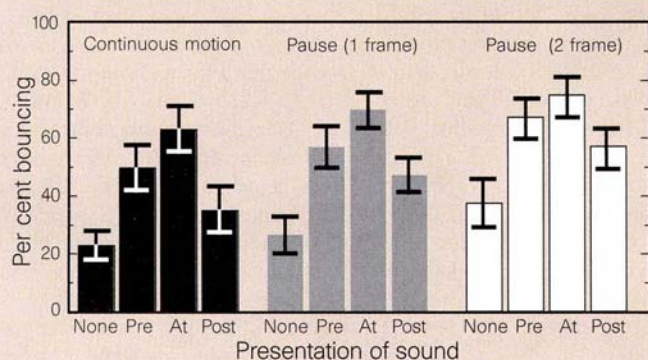


Figure 1 Percentage of reports of stimulus bouncing. In each trial, two brown disks (visual angle 0.5° , 3.5 cd m^{-2}) appeared on opposite sides of a computer display (white, 95 cd m^{-2}). Initially separated by 4.2° , the disks moved at 6° per s , coincided, then continued across the display. The trial ended when each disk had reached the other's starting position, and both disks disappeared from view (1.4 s). The sequence was viewed binocularly from a distance of 114 cm. Observers indicated whether the disks appeared to stream through or bounce off one another. Black bars, disks moved continuously; grey, one-frame and white, two-frame pause. Motion was accompanied by a brief sound 150 ms before (Pre) or after (Post) the disks coincided, or at the moment of coincidence. On one quarter of the trials, no sound was presented (None). Error bars, $\pm \text{s.e.m.}$ Full details of methodology and other results available on request from R. S.

1. Metzger, W. *Psychol. Forsch.* **19**, 1–49 (1934).
2. Gaver, W. W. *Ecol. Psychol.* **5**, 1–29 (1993).
3. Bertenthal, B. I., Banton, T. & Bradbury, A. *Perception* **22**, 193–208 (1993).
4. Sekuler, A. B., Sekuler, R. & Brackett, T. *Invest. Ophthalmol. Vis. Sci.* **36**, S50 (1995).
5. Sparks, D. L. & Groh, J. M. in *The Cognitive Neurosciences* (ed. Gazzaniga, M. S.) 565–583 (MIT Press, Cambridge, MA, 1995).
6. McIntosh, A. R., Cabeza, R., Lobaugh, N. & Houle, S. *Soc. Neurosci. Abstr.* **22**, 718 (1996).

scientific correspondence is intended to provide a forum in which readers may raise points of a scientific character. Priority will be given to letters of fewer than 500 words and 10 references.

Genius, scandal and the first seaclocks

The Quest for Longitude

Edited by William J. H. Andrews
Collection of Historical Scientific Instruments, Harvard University: 1996. UK distributor: Plymbridge. Pp. 437. \$75, £49.95

Longitude

by Dava Sobel

Fourth Estate: 1996. Published in the United States by Walker, \$19(hbk); Penguin, \$10.95 (pbk). Pp. 184. £12.99

Desmond King-Hele

The Quest for Longitude is a beautiful book, a sumptuous quarto with more than a hundred illustrations in colour and as many in black and white. The design has an air of affluence too: the main text occupies only the outer two-thirds of each page, leaving a generous inner column for the notes. The book is a pleasure to feel and to smell. Luxury oozes from its pages.

That is the image: what of the content? The book is a record of the papers presented during the Longitude Symposium at Cambridge, Massachusetts, in 1993, a date that marked the tercentenary of the birth of John Harrison, whose accurate clocks enabled mariners to measure their longitude at sea.

At the beginning of the eighteenth century, errors in estimating longitude at sea were often large enough to have fatal consequences. In 1714, the Parliament in London passed the Longitude Act, which offered a reward for anyone who devised an accurate and practicable method for determining longitude. Parliament was advised by an expert committee that included Sir Isaac Newton and Edmond Halley. For an accuracy of 0.5° in longitude on a voyage from England to the West Indies, the prize was set at £20,000, equivalent to more than £2 million (US\$3.3 million) today.

The prize attracted many "crack-brained longitude hunters": a dozen outlandish proposals are reviewed with gentle irony by Owen Gingerich in the book.

Two methods were regarded as serious contenders for the prize. The first was to produce a superbly accurate clock that would survive the harsh environment of a storm-tossed sailing ship. This would give the current time for a standard longitude, say 'Greenwich time' if the ship started from Greenwich. Local time at the ship's longitude could be established in the usual way: local noon is halfway between one observation of the Sun in the morning and a second at the same altitude in the afternoon (clouds permitting), with a correction for the equation of time. The longitude of the ship west of Greenwich is found by subtracting the local time from the Greenwich time.

Newton and most astronomers believed that such an accurate and rugged timepiece was impracticable, and they favoured the 'lunar-distance' method, described in the book by Derek Howse. This method relies on accurate prediction of the Moon's position among the stars. The actual distance of the Moon's limb from a star can be measured, and the 'Greenwich time' at which it was predicted to be there can then be deduced, after correcting for the effects of refraction and parallax. In practice the existing lunar predictions were not accurate enough, and the calculations required were daunting, being said to take four hours, with much danger of error.

Most established watchmakers believed that the required accuracy — about 2 seconds per day — was unattainable. But in a remote part of England, in north Lincolnshire, there lived "a unique and individual character" who was "able to think and work in isolation, free from the shackles of contemporary traditional thought", to quote from the paper by Andrew King.

This lone genius was Harrison (1693–1776), who spent more than 40 years perfecting the chronometer that told mariners exactly where they were and eventually won him

the prize. In the book William Andrews tells the story of Harrison's first three seaclocks, and Anthony Randall describes the development of the prizewinning instrument, H4. The awarding of the prize was in the hands of the Board of Longitude, which was dominated by astronomers biased in favour of the lunar-distance method. So Harrison had a hard time getting any interim payments in the early years. Fortunately for him he was helped first by Halley and then by the leading instrument-maker George Graham.

But the final payment of £8,750 was not made until 1773, when Harrison was 80, and then only after the intervention of King George III.

Although the book centres on Harrison, it covers a wide range of related topics. Four papers show how longitude connects with mathematics, navigation, cartography and history of science; and there are four more on chronometers after Harrison, well illustrated with elegant timepieces in colour.

This is an authoritative book with all the usual scholarly adjuncts, including extensive notes, a long bibliography and a full index.

An unexpected extra is a list of subscribers — a pleasant eighteenth-century touch. Inevitably



Harrison's prize-winning watch, H4: he designed the decorations around the dial himself.

there is duplication between related papers, but there are few errors or infelicities to mar the perfection of the volume.

Dava Sobel's *Longitude* was inspired by the same symposium, but it is as different as could be: it is a small book, by one author, without illustrations and almost without any numbers (except dates). This is the same story of Harrison's lifelong struggle with the mechanisms and the Board of Longitude, set out in a more dramatic fashion. The astronomers on the board were keen to see the money going towards better tables of lunar motion, and their bias against Harrison led to malpractices scandalous enough to make a good story.

The book is well written in American idiom and deserves its bestseller status. *Longitude* is usually regarded as boring, but here the action moves forward like a novel. A talking-book version is already on sale; an illustrated version is presumably on the way; and why not a television series or film? Before the hype gets out of hand, I should mention that there are some errors. The worst is in the sentence in parentheses on page 36: any sailors who calculate their longitude by this method risk being shipwrecked ("minutes and seconds" at the end should be "degrees and minutes").

Both books serve science well, and show its human face. The success of *Longitude* suggests that other stories of events in science, technology or medicine might be retold just as memorably. □

Desmond King-Hele worked as a space scientist for 40 years and is now writing a biography of Erasmus Darwin.

Backcloth of mystery

Relic, Icon or Hoax? Carbon Dating the Turin Shroud

by H. E. Gove

Institute of Physics Publishing: 1996. Pp. 336. £19.50, \$35

Robert Hedges

The origin of the Turin Shroud, with its unexplained image, is still the subject of doubts and questions. What is not doubted is the remarkable fascination the shroud holds for so many people. This unusual book attests the power of that fascination for the author, a physicist who played a principal role in developing the technique by which the shroud textile was eventually dated; and I dare say it will enthral those readers gripped by the minutiae of how the dating took place.

People of Christian faith may reasonably be expected to regard Christ's burial shroud as a supremely important object. But sanctifying the shroud not only cuts it off from the world, it makes the sacred susceptible to

scientific validation. In fact, the Roman Catholic Church has avoided offering such a hostage to fortune. Nevertheless, the pressure to sanctify relics is great; perhaps it recalls a distant time when all around us was regarded as holy. For others, the fascination appears more profane; they seem unable to leave open such questions as how the image was produced. Finally, there is a large measure of self-amplifying hype.

Against this heady backcloth of public interest, H. E. Gove had a key part in arranging the radiocarbon dating of the shroud. He seems to have kept extensive notes, and this book is a blow by blow, or rather telephone call by telephone call, account of his participation in the drama. It is largely autobiographical, while providing a journalistic, gossipy sort of history.

One feels that writing the book helped Gove to come to terms with his personal frustration about the way events unfolded. To have been foremost in developing the new dating technique, and to have been a prime agent for putting the Turin Shroud to the test, and then at a late stage to find that one's laboratory had been disinvited, was evidently deeply galling. It must explain some bitter personal remarks in this otherwise reasonably objective account.

The fascination of the shroud did indeed engender a level of competitive energy and hypocrisy rare in pure science. Both the account of the relationship between the radiocarbon-dating consortium and members of the Shroud of Turin Research Project, and the story of how the original selection of seven radiocarbon laboratories was reduced to three, provide interesting reading about some all-too-human aspects of scientific work. Gove writes that the elimination of his laboratory's participation was "inexplicable". This seems naive — the laboratories chosen were the ones with the greatest experience of archaeological dating, and all subsequent questioning of the date has been in terms of archaeological dating (validity and context of sample and questions of contamination).

Gove writes, more than once, that the technique was invented at his laboratory at the University of Rochester, New York. It seems poor judgement not to mention Nelson's work at McMaster University in Hamilton, Ontario, until an appendix (which credits Nelson with making the first carbon-14 measurements "virtually simultaneously" with Gove and his co-workers). But on factual events, I could find nothing I knew personally to be wrong, and am happy to accept the rest as accurate.

I am not sure at whom the book is aimed. Shroud aficionados will no doubt revel in the loving detail. But those wanting to understand the various issues — the state of evidence before the dating, the position of the ecclesiastical authorities, the scientific

basis of radiocarbon dating — may feel dissatisfied. The writing is lean and athletic, making it easy to enjoy reading the most trivial detail, and I found the pen sketches of my colleagues good fun. Even so, the relentless details of memoranda, meetings and telephone calls had stifled my interest long before I reached the account of the result of the dating. Gove covers the post-result period more briefly, perhaps because he was far less involved, although he does deal with most of the obstacles to accepting the date that were subsequently raised.

To convince the doubters, a much fuller and more energetic treatment is called for, and I feel this is a missed opportunity. The whole issue of the conflict in those who logically accept the scientific results and yet whose faith decrees those results to be in error has its own fascination, and deserves thoughtful and sympathetic treatment. But this is not that kind of book. □

Robert Hedges is at the Research Laboratory for Archaeology, University of Oxford, 6 Keble Road, Oxford OX1 3QJ, UK.

The short-range forces of nature

The Quantum Theory of Fields. Volume 2: Modern Applications

by Steven Weinberg

Cambridge University Press: 1996. Pp. 489. £32.50, \$47.95

John C. Taylor

Quantum field theory arose around 1930 as the rather inevitable result of combining quantum theory with special relativity. By 1950, quantum electrodynamics had been tamed, and the Lamb shift and the anomalous magnetic moment of the electron accurately calculated. These things and more were covered in volume 1 of Steven Weinberg's book, reviewed in *Nature* by S. S. Schweber (377, 396; 1995).

People naturally tried to apply quantum field theory to the other forces between sub-nuclear particles: the strong and the weak forces. These forces, in contrast to the electromagnetic ones, are of short range. At first there was not much success, and some physicists even doubted whether quantum field theory was the right framework in which to think about these nuclear forces. The breakthrough came towards the end of the 1960s when it was understood how to handle spin-one particles which differed from the photon in having mass and charge. These are the particles that are now known to mediate the short-range forces.

The problems were, first, how to avoid getting negative probabilities as a consequence of the minus sign in the Lorentz

metric ($x^2 + y^2 + z^2 - (ct)^2$); and, second, how to avoid an unacceptable increase of reaction rates at high energy. Each was solved by a combination of two theoretical ideas. The first of these, generalizing the gauge-invariance of electromagnetism, had been invented in 1954 by Yang and Mills and by Shaw. The second idea was the realization that the states of minimum energy may not be unique, in which case the physical vacuum 'chooses' one of them, thus having less symmetry than the Hamiltonian.

The first triumph of these new ideas was the unification of the weak forces with electromagnetism, and the prediction of the heavy, spin-one particles, the charged *W* and the neutral *Z*. In this work, as in so much else in the period, Weinberg himself played a leading part. The second success was the construction of a theory of strong interactions, based on a substructure of quarks with forces between them due to spin-one gluons.

One more technical advance was necessary to complete this picture. That was the realization that the strength of forces mediated by particle exchange depends on the energy of the process considered, and may decrease at high energy. This fact led people to believe that quarks and gluons may, in Weinberg's term, be "trapped", that is to say exist only within hadrons and not as truly free particles, but at the same time appear as nearly free to high-energy probes. It is this work of the second half of this century that is the subject of volume 2. Weinberg calls it *Modern Applications*, but it seems to me that there are plenty of principles as well as applications.

It is a majestic exposition. The two volumes are structured in a logical way. Everything is explained with incisive clarity. Weinberg always goes to the heart of any argument, and includes many things that cannot be found elsewhere in the literature. Often I found myself thinking: "Ah! Now I understand that properly."

Will this be the last book on 'classical' quantum field theory? Is there any more to be said? Certainly there are topics that Weinberg does not include, such as quantum theory on a space-time lattice (as a limiting process to define field theory). But apart from technical things such as this, does the future lie beyond field theory with supersymmetry, supergravity and string theory? Weinberg says that the first two of these may form the subject of volume 3.

I find it hard to imagine a better treatment of quantum field theory than Weinberg's. All serious users of the subject will want to have these two volumes on their shelves. □

John C. Taylor is in the Department of Applied Mathematics and Theoretical Physics, Cambridge University, Silver Street, Cambridge CB3 9EW, UK.

In retrospect chosen by Steve Jones

The Government Inspector

by Nikolai Gogol
(1836)

In pre-Revolutionary Russia, 14 levels of excellence were needed to reflect the nation's diversity of worth: yes, from Governor General to State Counsellor Second Class, each was indispensable. To move up a grade was man's sole purpose. In that pursuit all else was allowed to languish.

The recent Research Assessment Exercise did the same for British science. Thousands of hours of effort by the finest judges, tens of thousands of scientific papers masterfully ranked, and no suggestion of anything but fairness, probity and honour. The exercise had to make do, though, with a mere seven degrees of merit (the highest decorated with a star of Tsarist brilliance). As only those in departments given government sanction are to be allowed to pursue a research career, to gain the right grade has become as important as it was in Russia.

Nikolai Gogol's play *The Government Inspector* has, for a work written in 1836, a curiously modern air. Its plot is simple. An impatient St Petersburg, concerned that outlying provinces are failing to obey orders, sends out officials to bring them into line. A distant town is thrown into panic. It tries to disguise its weaknesses: patients are discharged from the hospital to reduce overcrowding; teachers who question the official line are sacked; and everything not productive is hidden away. At first the strategy fails, but, fortunately, it seems that the inspector is open to persuasion. Offer him what he wants and all will be well.

Any academic recognizes the plot. What modern head of department can fail to see himself in the mercenary and egotistical mayor, Dhumanovsky? "He has grown old in the service, and in his own opinion is far from being a fool... He has become a tyrant, but this is not due to any innate malice or urge to tyranny. It is inspired only by the desire to grab whatever he sets eyes on. The thought of all the things he might acquire and the dread of missing anything have become with him habits too deeply rooted ever to be overcome."

As the mayor becomes persuaded that his town will earn a high grade — a five, perhaps even a five-star! — a fascinating prospect opens. He may be promoted and, at last, move to the centre of power: "It would be fine to be a general! And wear ribbons and a sash over the shoulder! Nobody else will get a look in; they will all have to wait. Some poor wretch of a mayor will stand when I sit in comfort!"

The story is familiar. As the winners in the Research Assessment Exercise, like Tsar

Nicholas' courtiers, pin that extra star onto each other's glittering chests do they, too, pause to think of the distant regions in which the serfs of science have learned that, by official whim, they are to starve? Probably not: after all, who cares about those stupid enough to make a career in the provinces when academia has an Imperial Household to support?

Gogol's plot, though, has a twist. The supposed inspector is in fact a buffoon whose

Any academic recognizes the plot. What modern head of department can fail to see himself in the mercenary and egotistical mayor, Dhumanovsky?

only qualification is his own self-importance. The play ends in general dismay with the arrival of the real inquisitor and the sickening realization that all has collapsed and it is too late to do anything about it.

Russia's policy of central control with its matching corruption, intellectual and otherwise, lasted until long after the Revolution. So smoothly did it work that it persisted as the Soviet system itself fell into ruin. For those involved it made perfect sense. The logic, though, was completely internal. To any unbiased observer it was a dishonest shambles.

It would be quite wrong to use such terms to describe any British institution. Since the last Research Assessment Exercise the average score of those appraised has gone up by half a point. Perhaps the Dhumanovskys of the academic world are learning to manipulate the system as one five-year plan succeeds another. More likely, though, the exercise really does deserve the statement made by one of its officials, with the air of Stalin hailing yet another record year on the collective farms: "We congratulate universities and colleges on these results. The real winner is the United Kingdom."

Gogol's description of his play was: "In *The Government Inspector* I wished to turn all into ridicule. The real impression produced was that of fear. Through the laughter, the spectator feels only bitterness and sorrow."

What better description of a literary classic with significance for science today?

Steve Jones is at University College London, WC1E 6BT, UK.

Dubious revolution

The Scientific Revolution

by Steven Shapin

University of Chicago Press: 1996. Pp. 225.

\$22.95, £15.95

William Shea

The Scientific Revolution, meaning roughly the period that runs from Copernicus to Newton, is so deeply entrenched in the literature that it is hard to believe that it was only given broad currency some 40 years ago in a book of that title by A. Rupert Hall. For nineteenth-century historians, the great revolutionary changes that catapulted Europe into the modern age were the Reformation and the Renaissance. For Hall, the breakthrough was the result of the twin advance of mathematical physics and factual discovery that began in the sixteenth century.

The shift may have been occasioned by a lessening of interest in religion and art, but it may also have been caused by growing concern over the status of modern science. When a society is perfectly satisfied with its method of knowing, it is not greatly exercised about the way it was first acquired. As long as science was considered to be the embodiment of rationality, its history was less an examination of actual events than a celebration of heroes and a quest for anticipations.

The belief that science was inevitably progressive was given canonical formulation by George Sarton in *The Study of the History of Science*, first published in 1936 and made popular in a paperback edition several years later. This view was based on the following premises. Definition: science is systematized positive knowledge or what has been taken as such at different ages and in different places. Theorem: the acquisition and systematization of positive knowledge are the only human activities that are truly cumulative and progressive. Corollary: the history of science is the only history that can illustrate the progress of mankind. In fact, progress has no definite and unquestionable meaning in fields other than science.

This naive view was echoed by philosophers of science who pointed out that consensus was possible in science but a forlorn hope elsewhere. They claimed this was the case because science dealt with matters of fact (as opposed to armchair theories) and resolved its disputes by invoking appropriate rules of inference instead of mere rhetoric.

Thomas S. Kuhn challenged the legitimacy of this rigid dichotomy between science and other kinds of knowledge in *The Structure of Scientific Revolutions* in 1962. He showed that the historical genesis of a theory is relevant to its content as well as its

social acceptability. As a result, the canons of inductive logic so laboriously elaborated by philosophers of science began to look like parlour games. The pendulum swung from high-minded logical rigour to trendy sociological determination. Science, once the pure light of rationality, became a garish sign that was turned on and off by concealed pulsations in society.

What the exponents of the old and the new view had in common was a simplistic philosophy and a selective memory. Neither had a genuine desire to understand what had happened in the past. In their hurry to draw a moral for the present, they steam-rolled their way through history and invented a mythical Galileo and a legendary Bacon.

Steven Shapin's book attempts to rise above the warring factions. He looks at seventeenth-century science with an eye for all the evidence, even when it leads him into places that are considered too murky by advocates of the linear progress of science or too rational by those who worship serendipity. His description of the Scientific Revolution is not arrived at by imposing a twentieth-century template on the seventeenth century, but by attending to the actual unfolding of the scientific process. The three chapters deal sequentially with what

was known about the natural world in the seventeenth century, how that knowledge was secured, and what purposes it was intended to serve.

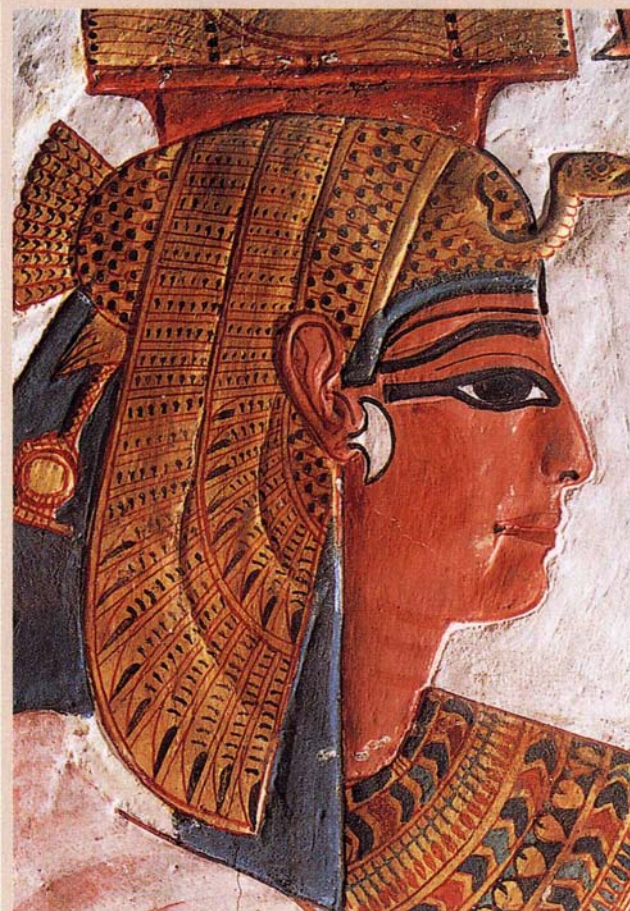
The first chapter covers topics included in most accounts of the Scientific Revolution, such as the Copernican theory and the mechanization of nature. The second and third chapters offer a sociological approach to the way the new learning was acquired and the goals for which it was intended.

Shapin offers a stimulating if controversial idea of how modern science was made and put to use. Many historians of science will feel that he does not do justice to the lasting contributions of Galileo, Boyle and Newton, but then, Shapin sees science as a system of provisional beliefs and not a growing body of truths about nature. Those who disagree with him will discover other ways of looking at the Scientific Revolution in the comprehensive and well-organized bibliographical essay at the end of the book.

Occasionally strident, but always well documented, this book will help scientists to understand why the Scientific Revolution is a more complex and more interesting phenomenon than many assume to be the case. □

William Shea is at the Institute of the History of Science, Université Louis Pasteur de Strasbourg, 7 Rue de l'Université, 67000 Strasbourg, France.

The art of burial in the land of the Pharaohs



Nefertari, the favourite Queen of Rameses II, stares out from the wall of her tomb in Egypt's Valley of the Queens. The tomb was sealed more than 3,000 years ago and was not discovered in modern times until 1904. When the exquisite paintings that cover its walls began to deteriorate, the tomb was revealed until 1986 when a nine-year conservation programme was begun. It has since reopened to the public. John K. McDonald tells the full story in his lavishly illustrated book *House of Eternity: the Tomb of Nefertari* (Thames and Hudson, £12.95).

A tension-based theory of morphogenesis and compact wiring in the central nervous system

David C. Van Essen

Department of Anatomy and Neurobiology, Washington University School of Medicine, 660 South Euclid Avenue, St Louis, Missouri 63110, USA

Many structural features of the mammalian central nervous system can be explained by a morphogenetic mechanism that involves mechanical tension along axons, dendrites and glial processes. In the cerebral cortex, for example, tension along axons in the white matter can explain how and why the cortex folds in a characteristic species-specific pattern. In the cerebellum, tension along parallel fibres can explain why the cortex is highly elongated but folded like an accordion. By keeping the aggregate length of axonal and dendritic wiring low, tension should contribute to the compactness of neural circuitry throughout the adult brain.

The central nervous system (CNS) comprises a diverse collection of neural structures, each with a distinctive shape and an intricate internal architecture. Questions about morphogenesis—how these structures attain their particular shapes during development—have intrigued neuroscientists for more than a century, but many mechanistic issues remain unresolved. Here I propose that mechanical tension, working against internally generated hydrostatic pressure, is a major driving force for many aspects of CNS morphogenesis. The clearest examples supporting this hypothesis are the cerebral cortex and cerebellar cortex, which in many species have extensive convolutions that allow a large surface area to fit within the available cranial volume. For example, the human cerebral cortex attains a surface area of about 1,600 cm², nearly three times what it would be in the absence of convolutions^{1,2}. For a given species, the folds have a consistent position in relation to identified cortical areas in some regions, but in other regions the pattern shows marked individual variability^{3,4}. Tension-based morphogenesis can account for the variability, as well as the consistency, of convolutions in terms of underlying patterns of connectivity between cortical areas.

Tension has previously been suggested to contribute to morphogenesis in the peripheral nervous system^{5,6} and in various non-neural cells and tissues^{7–9}. However, its relevance for CNS development has received surprisingly little consideration. The prospect of a major role for tension arises because of two basic anatomical characteristics of CNS tissue. First, many structures have pronounced anisotropies in the orientation of axons, dendrites and glial processes. If these processes are under tension, their springiness will make the tissue elastic, but the elasticity will not be uniform along all axes. Instead, the mechanical compliance should be lowest (resistance to stretching highest) along the axis of preferred orientation. Accordingly, tissue expansion should occur preferentially along axes that have greater compliance. The second characteristic involves projection asymmetries, in which the trajectories of processes arising or terminating in a given region are biased towards one side. One such example is the cerebral cortex, in which long-distance connections enter and leave the cortex exclusively through the underlying white matter. During cerebral growth, tension along axons acting together should pull strongly interconnected regions towards one another while allowing weakly connected regions to drift apart. The combined effects should keep wiring length short and overall neural circuitry compact.

Computer designers strive to place electronic components efficiently, yet invariably devote far more space to wiring than to

computation and memory combined¹⁰. Similarly, the nervous system devotes most of its space to non-synaptic wiring, even though minimizing wiring length has been proposed as an important design principle of neural architecture^{11–16}. One example of compact wiring (also called ‘component placement optimization’¹⁴) is the layout of areas across the surface of the cerebral cortex, as connections occur with high probability between adjacent areas but with low probability between distant areas^{14,17–19}. Here I extend this type of analysis to the third dimension^{14,20,21}, allowing the generality of compact wiring to be assessed.

Are neurons under tension?

The macroscopic mechanical properties of biological tissue depend on the active and passive mechanical characteristics of its microscopic constituents^{22,23}. For neural tissue, the most relevant structures are its elongated axons, dendrites and glial processes. Five characteristics relevant to morphogenesis have been demonstrated for the axon-like neurites that are extended by neurons and neuronal cell lines *in vitro*. (1) Neurites growing on an adhesive substrate generate substantial mechanical tension (stress)^{24,25}, averaging around 150 μ dyn for chick sensory neurons²⁶. Given a neurite diameter of 1 μ m or less (S. Heidemann, personal communication), this corresponds to an impressive 1% of the specific tension that can be exerted by mammalian skeletal muscle²⁷. (2) When a neurite is transiently stretched, its length increases in proportion to applied tension, indicating simple elastic behaviour²⁵. (3) When resting tension is fully released, a neurite initially shortens only slightly; if pushed further, it becomes wavy like a compressed rubber band²⁸. (4) Neurites display viscoelastic properties under sustained stretching, as the initially elevated tension relaxes passively to a lower level over a period of minutes²⁸. (5) Active elongation (towed growth) occurs when tension is maintained above a threshold level, and active retraction occurs when tension is fully released^{26,28}. Collectively, these five passive and active mechanical properties allow neurites to adjust their length by a negative feedback mechanism that tends to maintain a steady tension, much as a fishing line is reeled in or out to regulate tension.

The macroscopic mechanical properties of the brain *in vivo* are consistent with neurite properties observed *in vitro*. Brain tissue springs back to its original position after transient deformation, indicating that many cellular processes are elastic and under resting tension. Supporting this inference is the widespread occurrence of straight or smoothly curved neuronal processes, particularly in early development^{29,30}. Curved trajectories suggest tension coupled with

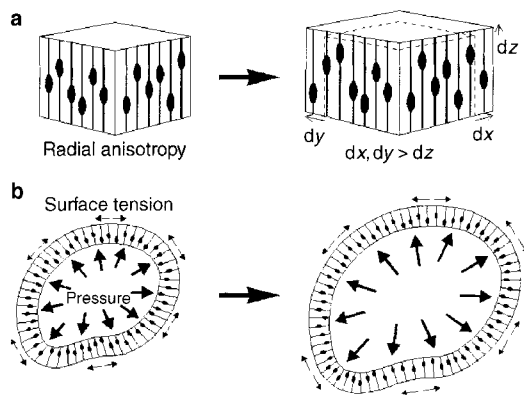


Figure 1 Mechanical factors contributing to anisotropic tissue growth. **a**, Preferential tangential growth of a radially anisotropic tissue (a schematic embryonic neuroepithelium). If cellular processes are under tension, the radial (vertical) axis would be mechanically stiffer than the tangential axes. If cells grow by exerting uniform pressure in all directions, expansion would occur preferentially along tangential axes, the path of least resistance ($dx, dy > dz$). This anisotropic expansion would occur if growth is mediated by passive visco-elastic tissue, and also if cells show active growth properties, as long as growth rates *in vivo* are proportional to tension, as they are *in vitro*²⁶. **b**, Balloon-like expansion of a quasi-spherical sheet (the embryonic neuroepithelium) surrounding a fluid-filled ventricular space that generates outward pressure (large arrows). This causes surface tension in the neuroepithelial wall (small arrows), and also compressive force along the radial axis, adding to the bias for tangential expansion arising from the radial anisotropy shown in **a**.

displacement by pressure from neighbouring axons, analogous to the arc on a fishing line caused by a breeze. There are also many processes with wavy or curled profiles (at least at the time of tissue fixation)^{29–31}, suggesting that tension is not universal. However, to play a role in morphogenesis, tension need only be present on many processes for much of the time, not on all of them at all times.

Neurons generally establish long-distance connections early in development. To cope with embryonic growth and shape changes, axons must therefore adjust their length continually, with some growing much faster than others and some actually shortening, for example as the cortex folds. Tension, working through passive viscoelasticity plus active growth and retraction, is an ideal feedback signal for regulating axonal length *in vivo*, as it does *in vitro*. This feedback mechanism evidently persists into adulthood, given that extrinsic forces such as tumours cause gradual tissue deformations without axonal breakage. A corollary requirement is that mechanical adhesion between pre- and postsynaptic partners must occur at CNS synapses so they do not come apart when under tension. Evidence for strong synaptic adhesiveness comes from the observation that mechanical homogenization of brain tissue yields a large pool of synaptosomes, which are subcellular components with pre- and postsynaptic membranes that remain tightly apposed³².

If neuronal and glial processes are under tension, what prevents them from all shortening concurrently, leading to a smaller brain that is fully relaxed? CNS tissue lacks a rigid structural framework to prevent such a collapse; tension must instead be counterbalanced by hydrostatic pressure to establish biological 'tensegrity'^{9,33}. All cells have an intrinsic pressure differential across their plasma membrane, resulting from active-transport mechanisms that regulate osmotic balance, but pressure can also arise from extrinsic sources.

Patterns of tissue growth

Morphogenetic shape changes involve interactions among three factors: the local forces that cells generate as they grow and migrate;

the mechanical properties of the immediately surrounding tissue; and any extrinsic forces arising from tension or pressure generated at a distance. First, consider a simple situation involving tissue that is mechanically isotropic (because its axons, dendrites and glial processes are randomly oriented) and is not subject to external forces. If all cells grow at similar rates, the tissue would expand equally in all directions. This may explain the compact, rounded shape of many subcortical nuclei, whose cellular architecture is relatively isotropic^{34,35}.

In contrast, tissue expansion should generally be non-uniform if the cellular architecture is anisotropic or if external forces are biased (Fig. 1). For example, Fig. 1a illustrates a radially anisotropic architecture in which cellular processes are elongated along the vertical (radial) axis. If these processes are under tension, the radial axis would be mechanically stiffer than the tangential axes. Generalized cellular growth would lead to preferential expansion along the path of least resistance, that is, in the tangential plane. This bias may contribute to the sheet-like expansion of several radially anisotropic structures, including the embryonic neuroepithelium³⁶, cerebral cortex³⁵, and retina³⁵. However, these structures also have a standing pressure differential across their surface^{37,38}, maintained by a steady production of cerebrospinal fluid (aqueous humor for the eye). As in a balloon, this internal pressure would cause surface tension, which stretches the sheet (Fig. 1b). Experimentally reducing intracerebral pressure³⁹ or intraocular pressure⁴⁰ reduces the rate of tangential growth, but the tissue remains a thin sheet, suggesting that both pressure and radial architecture contribute to anisotropic growth.

Not surprisingly, forces arising from surrounding non-neural structures can also affect the shape of the brain. For example, deformation of the skull arising from the premature closure of cranial sutures leads to corresponding changes in brain shape⁴¹. Morphogenetic forces operate in the other direction as well; in cases of hydrocephalus, for example, abnormal expansion of the brain has a marked effect on the size and shape of the skull.

Folding of the cerebral cortex

The cerebral cortex forms as a smooth sheet populated by neurons that proliferate at the ventricular surface and migrate outwards along radial glial fibres (Fig. 2a). But why does the cortical sheet remain smooth (lissencephalic) in some species, particularly those with small brains, yet become highly convoluted (gyrencephalic) in others, particularly those with large brains? The primary reason is that cortical surface area increases disproportionately with brain size. For example, in humans, cortical surface area is 1,700-fold greater than in shrews (~40-fold in linear dimensions), whereas cortical thickness is only sixfold greater⁴². Most of this bias is attributable to the aforementioned preference for tangential versus radial expansion. The remainder arises because the volume of neocortical grey matter, expressed as a percentage of total brain volume, increases with brain size, from about 13% for basal insectivores⁴³ to 50% for humans⁴². This results from differences in the duration of neurogenesis, which increases steeply with brain size for the cerebral cortex and less steeply for subcortical structures^{44–46}, leading to a systematic increase in the ratio of cortical to subcortical neurons. From this perspective, convolutions increase with brain size primarily because the expansion of the cortical sheet outpaces the minimal area needed to envelop the underlying cerebral volume.

When convolutions occur, what determines the spatial pattern of folding? Previous hypotheses about cortical folding have emphasized mechanisms intrinsic to the cortical grey matter^{3,47,48}, such as differential growth of different layers⁴⁹. However, folding patterns can be altered dramatically by prenatal lesions that perturb long-distance connections^{20,50,51}, suggesting that extrinsic factors are important.

Two observations suggest that tension along axons in the white

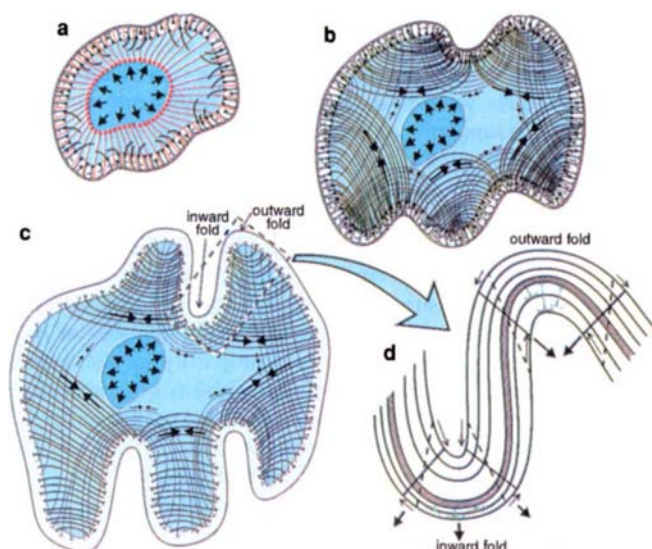


Figure 2 Tension-mediated folding of cerebral cortex. **a**, Early in development, neurons (black) migrate to the cortical plate along radial glial cells (red), differentiate and emanate axons. **b**, Many axons reach specific target structures before the onset of cortical folding. Tension (arrows) would pull strongly interconnected regions together and allow weakly interconnected regions to drift apart. **c**, This leads to outward folds that separate strongly interconnected regions, and inward folds that separate weakly interconnected regions. Connections with subcortical structures (not shown) may also influence cortical folding, although to a lesser degree because the tangential force components are smaller. **d**, Cortical folding causes shearing that tends to stretch the radial axis (broken lines). Compensatory tangential forces (small arrows) would tend to thicken the deep layers along outward folds and the superficial layers of inward folds, making their constituent cells (green) taller and thinner. The converse should occur in superficial layers of outward folds and deep layers of inward folds. Additional tangential force components associated with axons in the white matter (thick arrows) should enhance these effects on deep layers and counteract them in superficial layers.

matter is the primary driving force for cortical folding. First, the cortical sheet is physically tethered from only one side, initially by radial glial processes (Fig. 2a), followed shortly afterwards by connections between cortex (including the cortical subplate) and various subcortical nuclei⁵². Tension along inwardly directed processes would provide a cohesive force that works against intraventricular hydrostatic pressure to ensure that the cortical mantle remains tightly wrapped around the subcortical interior. Second, specific cortico-cortical projections are established early (Fig. 2b), as shown by neurons that send axons across the corpus callosum even while they are migrating to the cortex^{53,54}, and by topographically organized projections between visual areas in the macaque that are established while convolutions are forming³⁰ (see also ref. 20). Tension along obliquely oriented axonal trajectories between nearby cortical areas would generate tangential force components that tend to induce folds at specific locations in relation to areal boundaries (Fig. 2b, c). If developing CNS axons generate even a modest fraction of the specific tension measured *in vitro*, then populations of axons pulling together should have ample strength to cause folding of the highly pliable embryonic cortical sheet.

The cortex can fold in either of two polarities. In an outward fold, the crease is directed away from the interior, forming the crown of a gyrus and reducing the distance within white matter between opposite banks of the fold. Tension would pull strongly interconnected regions towards one another, forming an outward fold along their common border (Fig. 2b and c, heavy lines). In an inward fold, the crease is directed towards the interior, forming the fundus of a

sulcus. This slightly increases the distance within the white matter between opposite banks, so it provides no direct advantage for wiring length in the immediate vicinity. However, geometrical constraints require an inward fold between each pair of outward folds. In a general tug-of-war among many pathways competing for different folding patterns, sparse projections would generally be ineffective at resisting tissue displacements that force their axons to elongate. Consequently, outward folds should tend to occur between neighbouring areas that are only weakly interconnected (Fig. 2b and c, fine lines). Altogether, tension-induced folding should contribute to compact wiring for the cortex as a whole.

When a paperback book is folded, adjacent pages slide relative to one another. When the cortex folds, sliding between layers should also occur, but to a lesser degree because of stretching and shearing forces, which alter cellular morphology and the thickness of different layers. Along inward folds, cells in deep layers should be stretched tangentially and compressed radially, making these layers thinner. In contrast, along outward folds, cells in deep layers should be stretched radially, making these layers thicker (Fig. 2d). These predictions match closely the characteristic differences in cortical architecture of gyral versus sulcal regions of the adult cortex⁵⁵, suggesting that differential growth of cortical layers is a consequence, not a cause, of the forces that induce folding.

Compact cortical wiring

I analysed the compactness of cortical wiring and the relevance of tension-based morphogenesis to cortical folding in the macaque, whose convolutions are stereotyped and whose cortico-cortical connections have been intensively studied¹⁷. The location of several

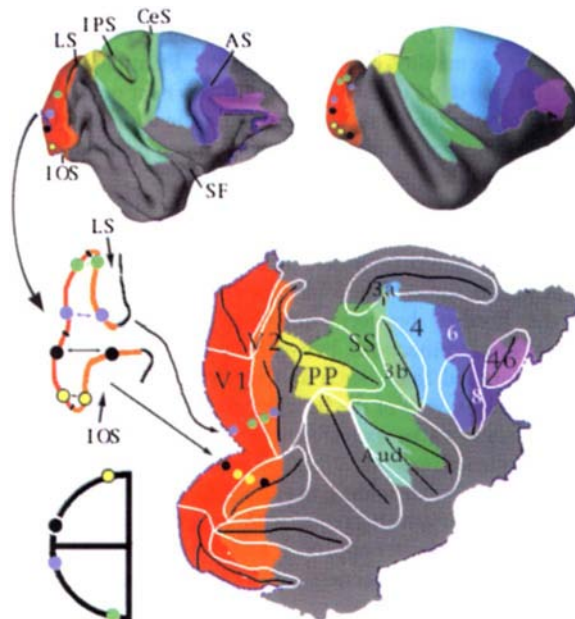


Figure 3 Compact wiring of cerebral cortex in the macaque, revealed by folding patterns in relation to area boundaries and connection patterns. Lateral views of the right hemisphere are shown before (upper left) and after (upper right) extensive smoothing. A cortical flat map^{17,56} (lower right) includes area boundaries and connection patterns. A parasagittal slice through occipital cortex (left centre) and on the two-dimensional cortical map, based on reported topographic organization of areas V1 and V2^{57,6}. The connectivity and folding patterns indicate that wiring is compact (see Table 1 and text). Abbreviations: AS, arcuate sulcus; Aud, auditory; CeS, central sulcus; IOS, inferior occipital sulcus; IPS, intraparietal sulcus; PP, posterior parietal; SS, somatosensory; SF, Sylvian fissure.

hypothesis

well-studied cortical areas or functionally related sets of areas in the macaque in relation to major cortical folds is shown in Fig. 3. The upper panels show a computerized reconstruction of the right hemisphere, viewed in its original configuration (upper left), and after computational smoothing to better reveal the interior of sulci (upper right). The lower right shows a cortical flat map⁵⁶ of the entire hemisphere (after introducing cuts that reduce distortions in surface area). The gyral and sulcal pattern on the cortical map is indicated by white lines for the crests of outward (gyral) folds and black lines for inward (sulcal) folds.

A striking example of compact wiring involves areas V1 (shown in red) and V2 (orange), which contain separate, mirror-image representations of the contralateral visual hemifield and are linked by massive reciprocal connections⁵⁷. In the intact hemisphere the two representations are brought into close proximity and approximate register by outward folds running along the V1/V2 border^{20,57}. This is illustrated for points near the vertical meridian (green and yellow) and the horizontal meridian (blue and black). Topographically corresponding loci in V1 and V2 are close together in three-dimensional space, as shown on a parasagittal slice through the cortex (left centre), even though loci near the horizontal meridian are widely separated on the flat map and in the embryonic hemisphere before folding³⁰. The alignment between V1 and V2 maps is less precise medially, in the vicinity of the calcarine sulcus, but is still better than if the folding pattern were random.

Table 1 documents the excellent correlation between qualitatively assessed connection strengths and the polarity of folding that occurs close to the border between adjacent areas or regions identified in Fig. 3. Strongly interconnected regions are consistently separated by an outward fold, whereas weakly connected regions are consistently separated by an inward fold. These regions collectively occupy more than half of the cortical surface, signifying that compact wiring is a widespread characteristic in the macaque. A survey of other species supports the generality of this conclusion. Examples include the consistent occurrence of the V1/V2 boundary near outward folds in humans³⁸, cebus monkeys⁵⁹ and cats⁶⁰, and the consistent positioning of somatosensory and motor cortex on opposite banks of an inward fold in primates, carnivores and rodents³.

Compact wiring of a system as a whole does not imply that all individual pathways are as short as possible. Indeed, many individual pathways would be much shorter if the cortex were folded differently. Also, some of the folds shown in Fig. 3 do not run exactly along the borders between functionally defined subdivisions. Finally, strong connections between opposite banks are lacking for some outward folds, such as that in the interior of area V1 (along the medial wall of the hemisphere). However, each of these characteristics is to be expected when minimizing the aggregate length of connections in a highly interconnected network. In general, tension-based folding may necessitate some folds that are neutral or even disadvantageous with regard to local connection lengths in order to better approach a global minimum.

Tension-based morphogenesis also suggests a basis for individual variability in brain morphology. No two brains are identical in the

detailed pattern and position of convolutions, nor in the size and exact connectivity patterns of various cortical areas. For example, the surface area of V1 in the macaque varies by more than twofold across individuals, yet outward cortical folds consistently run close to the V1/V2 boundary⁶¹, as expected if folding is dominated by connections between V1 and V2. Elsewhere, where competing pathways are evenly balanced, small individual differences in the relative sizes of areas or the strengths of pathways could tilt the balance between two or more qualitatively different folding patterns (for example, one sulcus versus two in a given region). These are analogous to local minima in the overall 'energy landscape' that characterizes possible states of a system⁶². The number of local minima generally increases with the complexity of the system, suggesting a basis for the notably high variability of convolutions in the human brain⁴. A corollary is that whether a particular cortical area ends up on a gyrus or in a sulcus may be of little importance for the specific computations mediated by that area, as long as the system as a whole has attained a near-minimal aggregate wiring length (for a given set of connections and topological arrangement of areas across the cortical sheet).

What selective advantage, if any, is conferred by an optimal pattern of cortical folds compared to various suboptimal configurations (for example, a cortex that is equally convoluted but has randomly positioned folds)? This question is difficult to address experimentally, but the conceptual issue is important in evaluating compact wiring and tension-based morphogenesis from an evolutionary perspective.

Generalizations to other structures

Given the variety in cellular architecture and connectivity patterns that occur in different embryonic CNS structures, to what extent can tension-based morphogenesis account for the great diversity of shapes evident in the adult CNS? Examples involving the cerebellum and the retina suggest that the explanatory power of the hypothesis is quite broad.

The cerebellum has a distinctive dual anisotropy in its cellular architecture, in which Purkinje cell dendrites and ascending axons of granule cells are aligned parallel to the radial axis, whereas the large contingent of parallel fibres is aligned along a single axis in the tangential plane. If these processes are all under tension, the resistance to stretching would be relatively high along both the radial axis and the axis of parallel fibres, leaving the axis orthogonal to parallel fibres as a single path of least resistance (Fig. 4, inset). This can explain why the disc-shaped embryonic cerebellum grows preferentially along this axis into a ribbon that, when unfolded, is often tenfold or more longer than it is wide^{63,64}.

Why is the cerebellum much more convoluted than the cerebrum, despite its smaller size? This outcome is predictable from the fact that cerebellar cortex is much thinner than cerebral cortex and is wrapped around a small subcortical volume (few nuclei, minimal ventricular space, and little white matter because of the absence of cortico-cortical connections). The accordion-like configuration of the cerebellar sheet may reflect shearing forces that differ according

Table 1 Connection strength and folding polarity in macaque cerebral cortex

Areas	Connection strength	Folding polarity	Location of fold(s)	Reference
V1 ↔ V2	Strong	Outward	Lunate, calcarine, inferior occipital sulci	57
Somatosensory (1, 2, 3, 4, 7b, SII)	Strong	Outward	Postcentral gyrus, inferior parietal lobule	71–73
4 ↔ 6	Strong	Outward	Precentral gyrus	71, 74
8 ↔ 46	Strong	Outward	Precuneate gyrus	75, 76
3b ↔ 4	Weak	Inward	Central sulcus	71, 72
Visual ↔ somatosensory	Weak	Inward	Intraparietal sulcus	72, 77
Auditory ↔ somatosensory	Weak	Inward	Sylvian fissure	73, 78
6 ↔ 8	Weak	Inward	Arcuate sulcus	74, 76

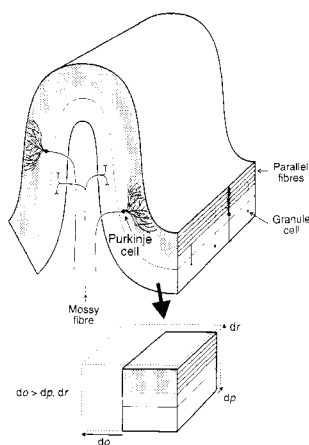


Figure 4 Dual anisotropies in the mammalian cerebellum. Purkinje cell dendrites and ascending axons of granule cells are aligned along the radial axis, and parallel fibres in the superficial layers are aligned along one tangential axis (mediolateral in many regions), jointly accounting for the ribbon-like elongation of cerebellar cortex (inset). Afferent fibres might bias the position at which folds occur if they preferentially distribute to opposite banks of outward folds, as shown hypothetically for a mossy fibre.

to the axis of folding. Folding occurs primarily along the axis of parallel fibres ('with the grain'), which allows parallel fibres to slide relative to one another without changing in length (Fig. 4). Folding 'against the grain' (orthogonal to parallel fibres) should be resisted because it would impose additional stretching of parallel fibres along each outward fold. Unlike cerebral cortex, the specific locations of cerebellar folds cannot be based on patterns of cortico-cortical projections, because such connections are altogether absent in cerebellar white matter. Undiscovered asymmetries in the connectivity of afferent inputs (mossy fibres and climbing fibres) might influence where folds occur, but a plausible alternative is that the positioning of cerebellar folds (as distinct from their orientation) is largely arbitrary with respect to connection patterns or functional subdivisions of the cerebellum.

In the retina, many species have a specialized foveal region that subserves high visual acuity⁶⁵. A prominent morphological characteristic of the fovea is a tangential displacement of retinal ganglion cells, away from the region of maximal cone density, which occurs

during late embryonic and early postnatal development⁶⁶. Before this displacement, ganglion cells initially direct their axons outwards from the centre of the presumptive fovea, and at some distance away these axons aggregate into fibre bundles (arcuate fascicles) that loop around to reach the optic disc⁶⁷ (Fig. 5a, b). If ganglion cell axons are physically anchored where they join the arcuate fascicles, tension along them would include a tangential component that pulls ganglion cell bodies away from the centre of the fovea, with bipolar and cone cell processes trailing because cone cell bodies are anchored at the outer retinal surface (Fig. 5c, d). Displacements should be largest in the centre of the fovea, where the ratio of ganglion cells to cones is maximal and where the tethering capacity of foveal cones is weakened by a postnatal reduction in diameter that is associated with an increase in visual acuity⁶⁶. Towards the periphery, each ganglion cell is anchored to progressively more cones (by intervening bipolar cells), which can explain the progressively closer alignment between ganglion cells and the cones they subserve (Fig. 5d). The reduction in retinal thickness in the foveal pit is thought to be functionally significant because it reduces light scatter and improves optical image quality where visual acuity is highest⁶⁵. Thus tension-driven retinal morphogenesis may have an adaptive role that involves more than just reducing wiring length.

Concluding remarks

In a classic analysis of growth and form, D'Arcy Thompson²² discussed how tension and pressure can interact with structural anisotropies and asymmetries to determine the shape of biological structures. He applied this perspective to a variety of peripheral body parts, and even to plants, but not to the brain. The present theory of tension-based morphogenesis of the CNS can be viewed as a natural, albeit belated, extension of his pioneering ideas. Although clearly speculative, my theory offers a simple and coherent explanation for many diverse aspects of CNS structure and development, and is already supported by numerous lines of evidence.

Four major areas merit further investigation. (1) It is important to determine the mechanical properties of CNS tissue, especially structures with anisotropic cellular architecture, and to measure the physical forces (tension and pressure) actually generated during morphogenesis. (2) A much wider range of species and structures should be examined to determine the generality of compact wiring in the adult, and the degree to which the pattern of development is consistent with tension-based morphogenesis. Of particular interest are structures such as the hippocampus and olfactory bulb, which

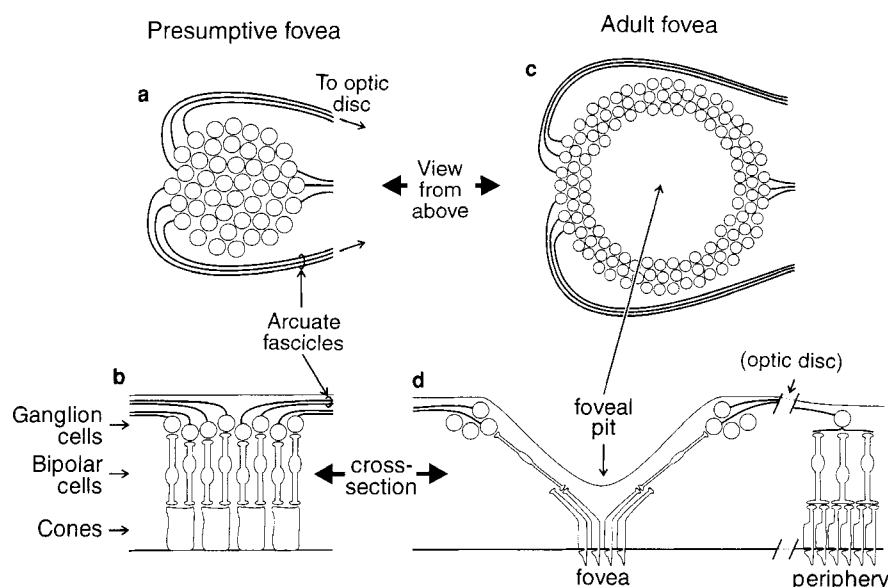


Figure 5 Tension-mediated formation of the fovea. **a**, Neonatal retinal ganglion cell axons radiating outward from the presumptive foveal region and joining into arcuate fascicles, viewed from above. **b**, Cross-sectional view at the same stage, showing ganglion cells positioned directly over the cones they subserve. **c**, Ganglion cell distribution in the adult fovea, showing the displacement away from the centre to form the foveal pit, viewed from above. **d**, Cross-sectional view, showing an offset between ganglion cells that are weakly tethered by cones in the fovea, but no offset for the strongly anchored ganglion cells in the periphery.

have distinctive architecture and connectivity patterns that may be responsible (by way of tension) for their unique shapes in the adult. (3) Experimental perturbations to distinguish between alternative models are needed. Do specific alterations in neural connectivity caused, for example, by congenital developmental abnormalities (such as lissencephaly^{68,69}), early surgical lesions^{50,51}, or genetic mutations⁷⁰, cause shape changes consistent with tension-based morphogenesis but not with other models? (4) Computer simulations can test whether the specific sequence of shape changes that occur during development of particular CNS regions can be replicated using plausible assumptions about forces and cellular dynamics, along with quantitative information about connectivity patterns. Collectively, these tests should reveal the degree to which

tension-based morphogenesis and compact wiring represent fundamental principles of neural development and function.

Morphogenesis entails an intricate choreographing of physical forces that cause differential tissue growth and displacement. Does this require an elaborate set of developmental instructions, transcending those needed to regulate the processes of neural proliferation, migration, axonal pathfinding, and synapse formation? If morphogenesis is driven largely by tension, the answer is no. Instead, the specificity of shape changes would largely be a by-product of factors that dictate the connectivity and topology of the underlying neural circuitry. This constitutes an efficient strategy for sharing the instructions that guide neural development. □

1. Drury, H. A. & Van Essen, D. C. *J. Neurosci* (Submitted).
2. Jouandet, M. et al. *J. Cogn. Neurosci.* **1**, 88–117 (1989).
3. Welker, W. in *Cerebral Cortex* (eds Jones, E. G. & Peters, A.) 3–136 (Plenum, 1990).
4. Ono, M., Kubik, K. S. & Abernathy, C. D. *Atlas of the Cerebral Sulci* (Thieme, Stuttgart, 1990).
5. Harrison, R. G. *Proc. R. Soc. Lond. B* **118**, 155–196 (1935).
6. Bray, D. *J. Cell Sci.* **37**, 391–410 (1979).
7. Odell, G. M., Oster, G., Alberch, P. & Burnside, B. *Dev. Biol.* **85**, 446–462 (1981).
8. Stopak, D. & Harris, A. K. *Dev. Biol.* **90**, 383–398 (1982).
9. Ingber, D. E. & Folkman, J. in *Cell Shape Determinants* (ed. Stein, W. D.) 3–31 (Academic, New York, 1989).
10. Mead, C. & Conway, L. (eds) *Introduction to VLSI Systems* (Addison-Wesley, Reading, MA, 1978).
11. Allman, J. & Kaas, J. *Brain Res.* **76**, 247–265 (1974).
12. Barlow, H. *Vision Res.* **26**, 81–90 (1986).
13. Chermiak, C. *Biol. Cybern.* **66**, 503–510 (1992).
14. Chermiak, C. *Trends Neurosci.* **18**, 522–527 (1995).
15. Cowey, A. Q. *J. Exp. Psychol.* **31**, 1–17 (1979).
16. Mitchison, G. *Proc. R. Soc. Lond. B* **245**, 151–158 (1991).
17. Felleman, D. J. & Van Essen, D. C. *Cereb. Cortex* **1**, 1–47 (1991).
18. Scannell, J., Blakemore, C. & Young, M. J. *Neurosci.* **15**, 1463–1483 (1995).
19. Young, M. *Nature* **358**, 152–155 (1992).
20. Dehay, C., Giroud, P., Berland, M., Killackey, H. & Kennedy, H. *J. Comp. Neurol.* **367**, 70–89 (1996).
21. Toga, A. W., Thompson, P. & Payne, B. A. in *Developmental Neuroimaging* (ed. Thatcher, R. W.) 15–27 (Academic, San Diego, 1996).
22. Thompson, D. *On Growth and Form* (Cambridge Univ. Press, 1917).
23. Wainwright, S. A. *Axis and Circumference. The Cylindrical Shape of Plants and Animals* (Harvard Univ. Press, Cambridge, MA, 1988).
24. Lamoureux, P., Buxbaum, R. E. & Heidemann, S. R. *Nature* **340**, 159–162 (1989).
25. Dennerll, T., Joshi, H. C., Steel, V. L., Buxbaum, R. E. & Heidemann, S. R. *J. Cell Biol.* **107**, 665–674 (1988).
26. Lamoureux, P., Zheng, J., Buxbaum, R. E. & Heidemann, S. R. *J. Cell Biol.* **118**, 655–661 (1992).
27. Burke, R. E. in *Handbook of Physiology—The Nervous System* (ed. Brooks, V. B.) 345–422 (Am. Phys. Soc., Bethesda, MD, 1981).
28. Dennerll, T. J., Lamoureux, P., Buxbaum, R. E. & Heidemann, S. R. *J. Cell Biol.* **109**, 3073–3083 (1989).
29. Cajal, S. R. *Studies on Vertebrate Neurogenesis* (Thomas, Springfield, 1960).
30. Coogan, T. A. & Van Essen, D. C. *J. Comp. Neurol.* **372**, 327–342 (1996).
31. Steinke, T. C. S. & Kirby, M. A. *Dev. Brain Res.* **74**, 151–162 (1993).
32. Whittaker, V. in *Handbook of Neurochemistry* Vol. 7 (ed. Lajtha, A.) 1–40 (Plenum, New York, 1984).
33. Buckminster Fuller, R. *Synergetics* (MacMillan, New York, 1975).
34. Morest, D. K. *Z. Anat. Entwickl.-Gesch.* **128**, 290–317 (1969).
35. Cajal, S. *Histologie du Systeme Nerveux de l'Homme et des Vertebres* (Maloine, Paris, 1911).
36. Sauer, F. C. *J. Comp. Neurol.* **62**, 377–405 (1935).
37. Miller, J. D., Peller, D. F., Pattisapu, J. & Parent, A. D. *Neurol. Res.* **9**, 193–197 (1987).
38. Pöll, W., Brock, M., Markakis, E., Winkelmueller, W. & Dietz, H. (eds Brock, M. & Dietz, H.) 188–194 (Springer, Berlin, 1972).
39. Desmond, M. E. & Jacobson, A. G. *Dev. Biol.* **57**, 188–198 (1977).
40. Coulombre, A. J. *J. Exp. Zool.* **133**, 211–225 (1956).
41. Enlow, D. *Facial Growth* (Saunders, Philadelphia, 1990).
42. Hofman, M. A. *Prog. Neurobiol.* **32**, 137–158 (1989).
43. Frahm, H., Stephan, H. & Stephan, M. *J. Hirnforsch.* **23**, 375–389 (1982).
44. Caviness, V. S. Jr, Takahashi, T. & Nowakowski, R. S. *Trends Neurosci.* **9**, 379–383 (1995).
45. Finlay, B. L. & Darlington, R. B. *Science* **268**, 1578–1584 (1995).
46. Rakic, P. *Trends Neurosci.* **18**, 383–388 (1995).
47. LeGrosClark, W. E. in *Essays on Growth and Form* (eds LeGrosClark, W. E. & Medawar, P. B.) 1–22 (Oxford University Press, London, 1945).
48. Barron, D. H. *J. Exp. Zool.* **113**, 553–573 (1950).
49. Richman, D. P., Stewart, R. M., Hutchinson, J. W. & Caviness, J. V. S. *Science* **188**, 18–21 (1975).
50. Goldman-Rakic, P. S. *Prog. Brain Res.* **53**, 3–19 (1980).
51. Rakic, P. *Science* **241**, 170–176 (1988).
52. DeCarlos, J. & O'Leary, D. J. *Neurosci.* **12**, 1194–1211 (1992).
53. Schwartz, M. L., Rakic, P. & Goldman-Rakic, P. S. *Proc. Natl. Acad. Sci. USA* **88**, 1354–1358 (1991).
54. Auladell, C., Martinez, A., Alcantara, S., Super, H. & Soriano, E. *Neuroscience* **64**, 1091–1103 (1995).
55. Bok, S. T. *Histology of the Cerebral Cortex* (Elsevier, Amsterdam, 1959).
56. Drury, H. A. et al. *J. Cogn. Neurosci.* **8**, 1–28 (1996).
57. Van Essen, D. C., Newsome, W., Maunsell, J. & Bixby, J. J. *Comp. Neurol.* **244**, 451–480 (1986).
58. Sereno, A. M. et al. *Science* **268**, 889–893 (1995).
59. Sousa, A. P. B., Carmen, M., Pinon, G. P., Gattass, R. & Rosa, M. G. P. *J. Comp. Neurol.* **308**, 665–682 (1991).
60. Tusa, R. J., Rosenquist, A. C. & Palmer, L. A. *J. Comp. Neurol.* **185**, 657–678 (1979).
61. Van Essen, D. C., Newsome, W. T. & Maunsell, J. H. R. *Vision Res.* **24**, 429–448 (1984).
62. Hopfield, J. J. & Tank, D. W. *Biol. Cybern.* **52**, 141–152 (1985).
63. Welker, W. I. *Arch. Ital. Biol.* **128**, 87–109 (1990).
64. Sultan, F. & Braltenberg, V. *J. Hirnforsch.* **34**, 79–92 (1993).
65. Polyak, S. *The Vertebrate System* (Univ. Chicago Press, 1957).
66. Hendrickson, A. E. & Yuodelis, C. *Ophthalmology* **91**, 603–612 (1984).
67. Kirby, M. A. & Steinke, T. C. *Vis. Neurosci.* **9**, 603–616 (1992).
68. Stewart, R., Richman, D. & Caviness, J. *Acta Neuropath.* **31**, 1–12 (1975).
69. Takada, K., Becker, L. & Chan, F. *Clin. Neuropathol.* **7**, 111–119 (1988).
70. Kuida, K. et al. *Nature* **384**, 368–372 (1996).
71. Jones, E. G., Coulter, J. D. & Hendry, S. H. C. *J. Comp. Neurol.* **181**, 291–348 (1978).
72. Pons, T. P. & Kaas, J. H. *J. Comp. Neurol.* **248**, 313–335 (1986).
73. Friedman, D. P., Jones, E. G. & Burton, H. *J. Comp. Neurol.* **192**, 21–41 (1980).
74. Barbas, H. & Pandya, D. N. *J. Comp. Neurol.* **256**, 211–228 (1987).
75. Barbas, H. *J. Comp. Neurol.* **276**, 353–375 (1988).
76. Barbas, H. & Pandya, D. N. *J. Comp. Neurol.* **286**, 353–375 (1989).
77. Seltzer, B. & Pandya, D. N. *Brain Res.* **192**, 339–351 (1980).
78. Galaburda, A. M. & Pandya, D. N. *J. Comp. Neurol.* **221**, 169–184 (1983).

Acknowledgements: I thank J. W. Lichtman, C. H. Anderson, S. Heidemann, P. Rakic, J. L. Price, T. Thach, J. Sanes, H. Burton, G. Christensen, T. Woolsey and others for suggestions and discussions; S. Danker for typing; and K. Fritts and H. A. Drury for help with illustrations. This work was supported by the NIH.

Correspondence should be addressed to the author (e-mail: vanessen@v1.wustl.edu).

Stochastic resonance in non-dynamical systems without response thresholds

Sergey M. Bezrukov*† & Igor Vodyanoy*‡§

* Division of Computer Research and Technology, National Institutes of Health, Bethesda, Maryland 29892-0580, USA

† St Petersburg Nuclear Physics Institute, Gatchina, Russia 188530

‡ Office of Naval Research, Europe, 223 Old Marylebone Road, London NW1 5TH, UK

§ University College London, Gower Street, London, UK

The addition of noise to a system can sometimes improve its ability to transfer information reliably. This phenomenon—known as stochastic resonance—was originally proposed to account for periodicity in the Earth's ice ages¹, but has now been shown to occur in many systems in physics and biology^{2–4}. Recent experimental and theoretical work has shown that the simplest system exhibiting 'stochastic resonance' consists of nothing more than signal and noise with a threshold-triggered device (when the signal plus noise exceeds the threshold, the system responds momentarily, then relaxes to equilibrium to await the next triggering event)^{4–6}. Here we introduce a class of non-dynamical and threshold-free systems that also exhibit stochastic resonance. We present and analyse a general mathematical model for such systems, in which a sequence of pulses is generated randomly with a probability (per unit time) that depends exponentially on an input. When this input is a sine-wave masked by additive noise, we observe an increase in the output signal-to-noise ratio as the level of noise increases. This result shows that stochastic resonance can occur in a broad class of thermally driven physico-chemical systems, such as semiconductor p–n junctions, mesoscopic electronic devices and voltage-dependent ion channels⁷, in which reaction rates are controlled by activation barriers.

In contrast to threshold behaviour, where subthreshold forcing does not generate any output signal, threshold-free systems are able to respond to input signals of arbitrary small amplitude. We consider here threshold-free systems whose output can be described by a random train of identical pulses with the probability of pulse generation exponentially depending on the input signal. In particular, we assume that the pulse generation rate is given by:

$$r(V(t)) = r(0) \exp(V(t)) \quad (1)$$

where $r(0)$ is the 'equilibrium rate' of the pulse generation and $V(t)$ is dimensionless voltage or some other external parameter. This equation describes a broad variety of 'kT-driven' physico-chemical systems in which reaction rates are controlled by activation barriers whose heights are dependent on an external parameter. The classical examples include thermionic emission (the Richardson equation⁸), electron transfer across semiconductor p–n junctions⁹, and the generalized Eyring's rate theory¹⁰—a foundation for kinetic phenomena description in contemporary physical and biological chemistry. Among modern examples are ion channels⁷ and mesoscopic electronic devices¹¹.

Figure 1 illustrates the model with two examples of the output pulse train for $V(t)$ equal to zero (on the left) and for $V(t)$ randomly changing in time (on the right). It is seen that at zero input voltage the output current, $I(t)$, is represented by randomly arriving pulses. The input voltage encodes its properties into the pulse train, so that large positive deviations of the input voltage from zero substantially increase the probability of pulse generation. As a mathematical

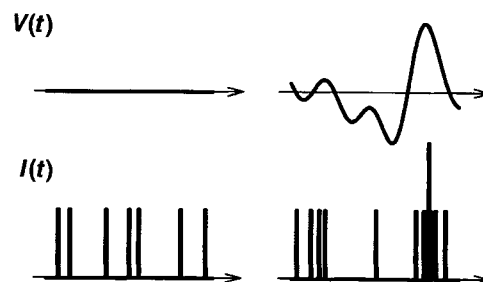


Figure 1 A time-dependent Poisson process is introduced via the voltage sensitivity of pulse generation rate. Randomly changing input voltage modulates the pulse arrival rate of an undisturbed stochastic process (tracks on the left) so that pulses start to group in time (tracks on the right). Large positive voltages increase the pulse rate to a degree where pulse overlapping is probable, and events with double (or multiple) amplitude appear in the output current.

object such a pulse train was introduced in 1955 by Cox who called it "doubly stochastic Poisson process"¹². Following Cox, we do not discuss here the origin of initial stochasticity that leads to the pulse generation randomness—it may have different origins for different systems described by our approach, including some kind of internal fluctuation.

To facilitate subsequent treatment, we assume that $V(t)$ is the sum of a slow zero-mean gaussian noise, $V_N(t)$, and a slow small-amplitude sine-wave signal, thus limiting ourselves to the adiabatic small-signal regime:

$$V(t) = V_N(t) + V_s \sin(2\pi f_s t) \quad (2)$$

where the signal amplitude $V_s \ll 1$ and the signal frequency f_s is much smaller than all other characteristic frequencies in the model.

By analysing the statistical properties of such a pulse train we will describe the following features of signal transduction in these systems: (1) threshold-free response—the ability to transfer small signals with a transduction coefficient independent of signal amplitude; (2) noise-facilitated signal transduction—the property to increase the output signal amplitude by addition of noise to the system input; (3) noise-induced improvement in the output signal-to-noise ratio—the existence of particular input noise levels that optimize the output signal quality.

For a random train of identical pulses the statistical properties of the process are entirely defined by the moments of pulse arrival. The shape of the pulse gives only a multiplicative 'form-factor' important only at high frequencies that are comparable to inverse pulse time. To describe the signal transduction properties of our model we use the standard language of power spectral density^{2–7}. At low frequencies around the signal frequency f_s , the power spectral density for this time-dependent Poisson process¹³ can be written in the form:

$$S_i(f) = 2Q^2 \langle r(V(t)) \rangle + 4(Qr(0))^2 \times \int_0^\infty \langle \exp(V(t)) \exp(V(t+\tau)) \rangle \cos(2\pi f\tau) d\tau \quad (3)$$

where Q is single pulse area, τ is the time delay and symbol $\langle \rangle$ implies averaging over the ensemble representing the stochastic process. The first term here describes the frequency-independent component of noise expected from an uncorrelated time-independent train (Poisson wave) of pulses, the second one accounts for the pulse rate modulation induced by the input signal and noise (equations (1) and (2)).

For a small-amplitude signal and gaussian noise, the first term is easily expressed through the noise second moment or the root-

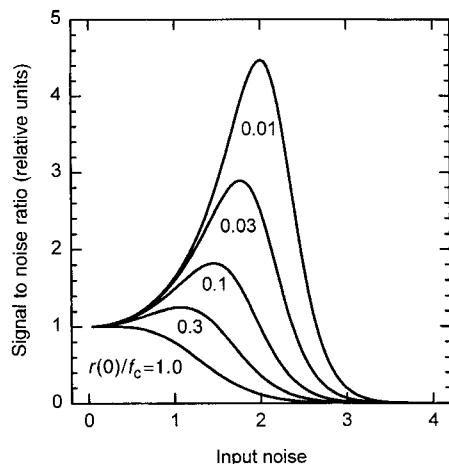


Figure 2 Output signal-to-noise ratio (SNR) can be improved by addition of the input noise. Small-signal adiabatic theory predicts the existence of optimal noise intensity corresponding to a maximum in SNR. The magnitude of the optimal input noise is sensitive to the 'equilibrium pulse rate', $r(0)$, and the noise corner frequency, f_c .

mean-square fluctuation σ :

$$2Q^2 \langle r(V(t)) \rangle = 2Q^2 r(0) \exp(\sigma^2/2) \quad (4)$$

where σ , as all other voltages here, is dimensionless. To calculate the second term, we use relations for the probability density of two-dimensional normal distributions of X and Y (ref. 14) introducing the random vector (X, Y) as $X \equiv V(t)$, $Y \equiv V(t + \tau)$. After some calculations, using the condition $V_s \ll \sigma$, we obtain:

$$\langle \exp(V(t)) \exp(V(t + \tau)) \rangle = \exp(\sigma^2(1 + \rho(\tau))) \quad (5)$$

For the band-limited 'white' noise with the corner frequency $f_c = (2\pi\tau_f)^{-1}$, the normalized autocorrelation can be written in the form:

$$\rho(\tau) = \exp(-\tau/\tau_f) + (V_s^2/2\sigma^2) \cos(2\pi f_s \tau) \quad (6)$$

At $f \ll f_c$, equation (3) becomes:

$$S_i(f) = 2Q^2 r(0) \exp\left(\frac{\sigma^2}{2}\right) + \frac{(Qr(0)\sigma^2)^2}{f_c} \exp(\sigma^2) \times \sum_{n=1}^{\infty} \frac{\sigma^{2n-2}}{n!n} + \frac{(Qr(0)V_s)^2}{2} \exp(\sigma^2) \delta(f - f_s) \quad (7)$$

Here, the first term on the right-hand side accounts for the noise expected from the time-independent train of pulses with the rate of $r(0) \exp(\sigma^2/2)$. The second term represents the external input voltage noise, $V_N(t)$, transduced to the output. It includes not only a small-signal part of the noise transduction but also contributions from the crosstalk between different spectral noise components. Signal transduction at $f = f_s$ is described by the term containing a delta-function. It shows that the power of the output signal grows exponentially with σ^2 and is finite at zero input noise. A d.c. component of the output is ignored.

The last two terms of equation (7) reflect the coding of the input stimulus (signal plus noise) in the pulse train. If the 'equilibrium rate' $r(0)$ were not disturbed at all, that is, if σ and V_s were zeros, these terms would vanish and would not contribute to the system output. The second term goes away also for the input noise with negligible correlations—in this case σ is held constant, but the noise corner frequency is increased to an extent where $f_c \gg r(0)\sigma^2$.

Output signal-to-noise ratio, SNR, is now easily obtained from the left part of equation (7) as the ratio of its last term to the

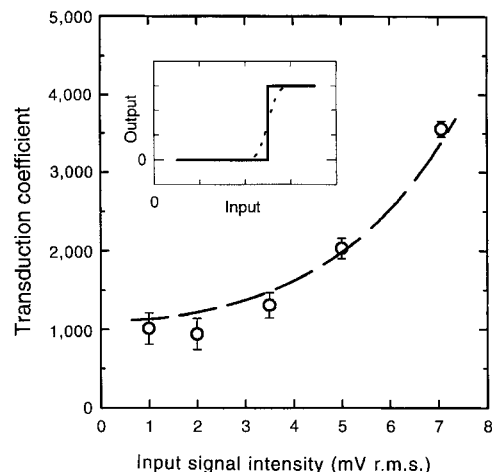


Figure 3 Signal transduction predicted by the model at zero input noise as a function of input signal intensity (dashed line) compared to those for threshold systems from electronics¹⁶ (solid line) and biology¹⁷ (dotted line) shown in the inset. The threshold-free character of the model is clearly seen. The points show the gain ratio measured as described in ref. 7 with the parallel array of alamethicin ion channels reconstituted in a planar lipid bilayer at 110 mV. The interrupted line is calculated for $kT/ne = 3.3$ mV.

first two:

$$\text{SNR} = \frac{\frac{V_s^2 r(0)}{2\Delta f_A} \exp\left(\frac{\sigma^2}{2}\right)}{2 + \frac{r(0)}{f_c} \sigma^2 \exp\left(\frac{\sigma^2}{2}\right) \sum_{n=1}^{\infty} \frac{\sigma^{2n-2}}{n!n}} \quad (8)$$

where Δf_A stands for the unit-frequency window width of the spectrum analyser that is to be used in measurements. It is seen that, except for the trivial dependence on the input signal amplitude (V_s) and the details of measurement technique (Δf_A), the SNR can be controlled by the noise intensity (σ), its frequency width (f_c), and 'equilibrium pulse rate' ($r(0)$).

Figure 2 shows the signal-to-noise ratio at different values of $r(0)/f_c$ as a function of input noise intensity. In the figure, the SNR is normalized to its magnitude at $\sigma = 0$. It can be seen that external input noise can improve the output signal quality. Noise intensity corresponding to a maximum in the output SNR increases with f_c but decreases with $r(0)$. Interestingly, however good the initial statistics (large $r(0)$), the output signal quality can be further improved if (and only if) the bandwidth of input noise is high enough for the condition $f_c > r(0)$ to hold.

It should be noted here that for small and slow signals the results of our calculations are exact. The presented model is applicable to a wide variety of systems with exponential statistics. In the case of biological ion channels the exponential statistics are due to an interaction between the part of channel molecule containing so called 'gating charges'¹⁵ and the electric field applied across the membrane in which this molecule is embedded. For such voltage-dependent ion channels Q becomes the total charge transported through a single channel during its conductive state, and all voltages in the problem are now expressed in units of kT/ne , where ne is an effective gating charge of the channel⁷.

Figure 3 shows signal transduction predicted by our model (dashed line) with experimental data obtained for voltage-dependent ion channels (data points) and demonstrates its threshold-free character. To highlight the qualitative difference, the solid and dashed lines in Fig. 3 inset describe examples of two threshold devices taken from electronics and biology: they demonstrate

the output signal of a level-crossing detector (adapted from ref. 16 for the case of zero external noise) and a nerve cell as a function of the input signal amplitude. The nerve cell is represented by the 'Hodgkin-Huxley axon' in the presence of electrical noise¹⁷ whose magnitude determines the width of the threshold transition. It is seen that at zero external noise, the threshold devices are silent at small signals, whereas voltage-dependent ion channels transduce them with a finite coefficient. □

Received 15 July; accepted 22 November 1996.

1. Benzi, R., Sutera, S. & Vulpiani, A. *J. Phys. A* **14**, 1453–457 (1981).
2. Bulsara, A. R. & Gammaitoni, L. *Phys. Today* **49**, 39–45 (1996).
3. Wiesenfeld, K. & Moss, F. *Nature* **373**, 33–36 (1995).
4. Moss, F., Pierson, D. & O'Gorman, D. *Int. J. Bifurc. Chaos* **4**, 1383–1397 (1994).
5. Wiesenfeld, K., Pierson, D., Pantazelou, E., Dames, C. & Moss, F. *Phys. Rev. Lett.* **72**, 2125–2128 (1994).
6. Gingl, Z., Kiss, L. B. & Moss, F. *Europhys. Lett.* **29**, 191–196 (1995).
7. Bezrukov, S. M. & Vodyanov, I. *Nature* **378**, 362–364 (1995).
8. Nye, J. F. *Physical Properties of Crystals* 236–240 (Clarendon, Oxford, 1976).
9. Benedict, R. R. *Electronics for Scientists and Engineers* 80–85 (Prentice-Hall, Englewood Cliffs, 1976).
10. Glasstone, S., Laidler, K. J. & Eyring, H. *The Theory of Rate Processes* 1–27 (McGraw-Hill, London, 1941).
11. Reznikov, M., Heiblum, M., Shtrikman, H. & Mahalu, D. *Phys. Rev. Lett.* **75**, 3340–3343 (1995).
12. Cox, D. R. *J. R. Statist. Soc. B* **17**, 129–164 (1955).
13. Cox, D. R. & Lewis, P. A. W. *The Statistical Analysis of Series of Events* 28–29, 102–112 (Methuen, London, 1966).
14. Rice, S. O. in *Selected Papers on Noise and Stochastic Processes* (ed. Wax, N.) 133–294 (Dover, New York, 1954).
15. Hille, B. *Ionic Channels of Excitable Membranes* 54–47, 372–503 (Sinauer Assoc., Sunderland, 1992).
16. Jung, P. *Phys. Lett. A* **207**, 93–104 (1995).
17. Lecar, H. & Nossal, R. *Biophys. J.* **11**, 1048–1067 (1971).

Acknowledgements: We thank V. A. Parsegian for discussions and encouragement at all stages of this work; G. Weiss and M. Taggi for consultations; V. Aguilera, B. Altshuler, C. Houdre, S. Leikin, R. Nossal, R. Podgornik and H. Strey for their suggestions and comments on the manuscripts. This work was supported by the US Office of Naval Research.

Correspondence should be addressed to S.M.B. (e-mail: bezrukov@helix.nih.gov).

Template-directed colloidal crystallization

Alfons van Blaaderen^{*}, Rene Ruel[†] & Pierre Wiltzius[†]

^{*} FOM Institute for Atomic and Molecular Physics, Postbus 41883, 1009 DB Amsterdam, The Netherlands, and Van't Hoff Laboratory, Debye Institute, Utrecht University, Postbus 80051, 3508 TB Utrecht, The Netherlands

[†] Lucent Technologies, Bell Laboratories, 700 Mountain Avenue, Murray Hill, New Jersey 07974, USA

Colloidal crystals are three-dimensional periodic structures formed from small particles suspended in solution. They have important technological uses as optical filters^{1–3}, switches⁴ and materials with photonic band gaps^{5,6}, and they also provide convenient model systems for fundamental studies of crystallization and melting^{7–10}. Unfortunately, applications of colloidal crystals are greatly restricted by practical difficulties encountered in synthesizing large single crystals with adjustable crystal orientation¹¹. Here we show that the slow sedimentation of colloidal particles onto a patterned substrate (or template) can direct the crystallization of bulk colloidal crystals, and so permit tailoring of the lattice structure, orientation and size of the resulting crystals: we refer to this process as 'colloidal epitaxy'. We also show that, by using silica spheres synthesized with a fluorescent core^{12,13}, the defect structures in the colloidal crystals that result from an intentional lattice mismatch of the template can be studied by confocal microscopy¹⁴. We suggest that colloidal epitaxy will open new ways to design and fabricate materials based on colloidal crystals and also allow quantitative studies of heterogeneous crystallization in real space.

Epitaxial growth of a thin crystalline layer onto a template consisting of an oriented single crystal is an important process in the development of electronic devices. For instance, in molecular

beam epitaxy careful control over reagent flow and temperature make it possible to achieve layer-by-layer growth of oriented crystal structures that can even be slightly out of equilibrium¹⁵. We have extended these ideas of template-directed crystallization to colloids, which are the ideal building blocks for photonic applications owing to their submicrometre sizes, self-organization and the possibility of chemically tailoring their properties. As a template we used a 500-nm-thick fluorescent polymer layer, with holes made with electron beam lithography (Fig. 1A). This thickness is close to the particle radius (525 nm) of the fluorescent silica spheres (fluorescent core: 200 nm). The interparticle forces are made hard-sphere-like by matching the refractive index of the solvent mixture to that of the spheres, minimizing van der Waals forces, and by increasing the ionic strength to such values that double-layer repulsion occurs over distances much smaller than the particle radius. Controlled layer-by-layer growth was achieved by slow sedimentation of the spheres onto the substrate from a volume fraction of particles well below the 50% volume fraction of homogeneous crystallization of hard-spheres^{7–10}.

Experimentally, hard-sphere-like colloidal dispersions are known to crystallize with a random stacking of close packed planes^{7–10,16}. Theoretically, it is not clear what the thermodynamic equilibrium phase is; also, computer simulations have not been able to determine whether face-centred cubic (f.c.c., with stacking order ABC), hexagonal close packed (h.c.p., stacking ABA) or random stacking is favoured¹⁷. It is known, however, that thermodynamically these structures have almost the same free energy. As suggested by others¹⁶, we found in this work that crystals that form through sedimentation have a random stacking with the close-packed planes perpendicular to gravity. Strictly speaking, a random stacked structure of close-packed planes is not a crystal, because of the randomness in one crystallographic direction. However, when a colloidal crystal was grown normal to a pattern of holes commensurate with the (100) plane of a f.c.c. crystal (Fig. 1A inset), a pure f.c.c. crystal was formed (Fig. 1B, C). By use of light scattering, we found that the f.c.c. crystal extended as far as the sedimented layer is thick (several millimetres). Also, the single crystals grew as large as the size of the pattern.

The reason for the formation of the f.c.c. crystal is that the (100) face can induce a three-dimensional structure: there is only one possible way the second layer can be placed on the holes created by the first layer. In other words, there is no twinning possibility along this growth direction (twinned crystals share a common set of atoms, but have a different orientation of their (otherwise similar) lattices). A sphere dropping into a hole 500 nm deep loses 0.6 *kT* in gravitational energy, where *k* is Boltzmann's constant and *T* the absolute temperature. Apparently, the small increased likelihood of a square symmetry as induced by the pattern shown in Fig. 1 is enough to start of the crystal growth with the (100) plane instead of the (111) hexagonal plane which is the densest crystal plane. For the growth of the crystal, the balance between diffusion and sedimentation is important. This balance is described by the Peclet number $Pe = m_b g R / kT$, where *m_b* is the buoyant mass of a particle with radius *R* and *g* is the gravitational acceleration. *Pe* is defined as the ratio of the time it takes a particle to diffuse over a distance *R* and the time it takes to settle over the same distance; in this work *Pe* ≈ 1. For the crystal layers close to the template, the situation is very different at the end of the growth. Here the weight of several thousand layers exerts such a pressure that osmotic pressure can only balance this at a crystal volume fraction experimentally indistinguishable from close packing, 0.74.

The growth of macroscopic f.c.c. single crystals on the (110) plane, which is even less dense than the (100) plane and also has no twinning directions, was also possible. These results make it likely that the growth of pure hexagonal close-packed (h.c.p.) crystals on a non-dense h.c.p. plane that has no associated twinning direction will be feasible as well.

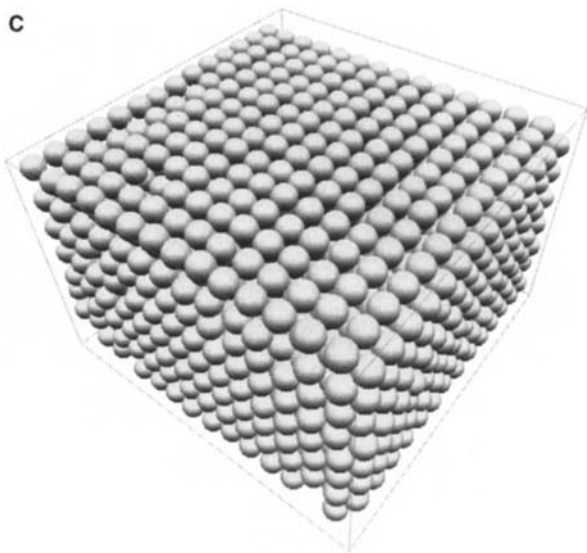
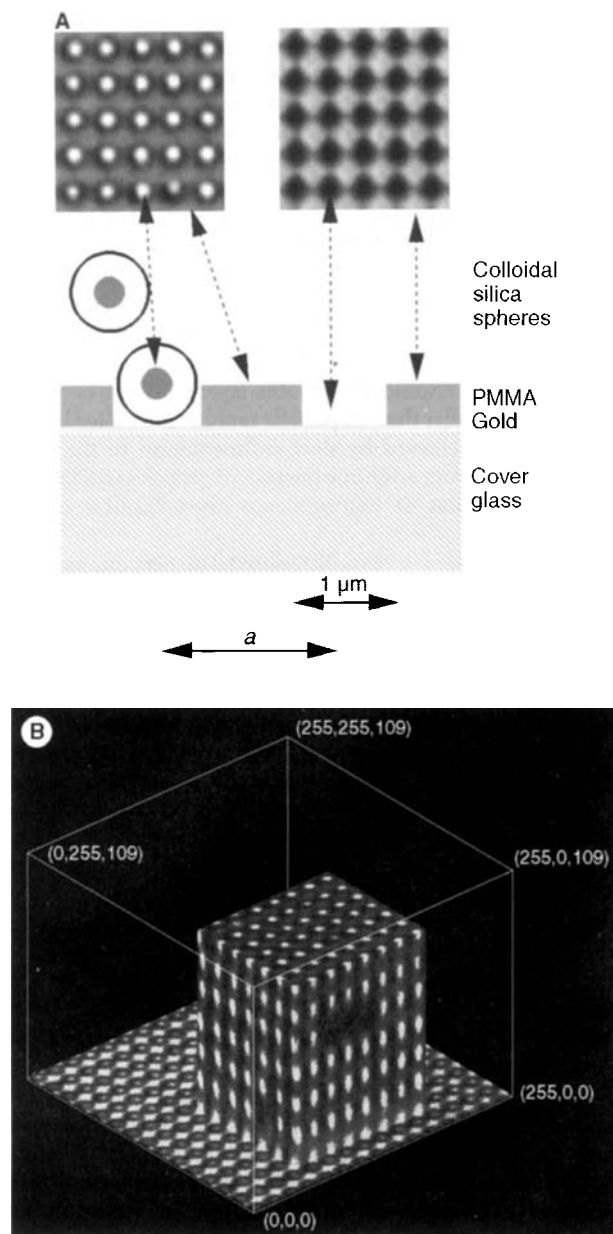


Figure 1 Face-centred cubic (f.c.c.) hard-sphere-like colloidal crystals grown on a (100) f.c.c. template created in a polymer layer. **A**, Schematic diagram of the colloidal epitaxy procedure (bottom) and two confocal micrographs (top) showing the fluorescently labelled polymer template with (left) and without (right) a first layer of spheres. **B**, Cut-out of a three-dimensional stack of confocal sections ($256 \times 256 \times 120$ voxels, three-dimensional picture elements, $16.5 \mu\text{m} \times 16.5 \mu\text{m} \times 10.5 \mu\text{m}$) on top of the polymer template. **C**, computer-generated picture of the experimental data set shown in **B** and with the sphere positions obtained as described in ref. 14. Methods: The silica spheres were dispersed in a refractive-index-matched mixture of water and glycerol (16 wt% glycerol) with 0.01 M LiCl and had starting volume fractions of 1% and 5%; no differences were found in this range of concentration. (The total radius of the silica spheres was 525 nm, of which the fluorescent core comprised 200 nm.) The dyed (pyromethene 580, 10^{-3} wt%) polymer (polymethylmethacrylate, PMMA) was spin-coated (500-nm thick) on a cover slip (no. 1; $170 \mu\text{m}$ thick) on which a thin layer of gold (5 nm) was sputtered. The pattern of holes (400×400) was created with electron beam lithography. The fluorescence confocal micrographs were made with a Multiprobe 2001 (Molecular Dynamics) confocal apparatus; see ref. 14 for further details. The distance of closest approach of the spheres was 1,105 nm in a glass and 1,110 nm in a crystal after full sedimentation. These values are close to the distances reported in 0.1 M dimethylformamide¹⁸ and show that the colloidal crystals are almost completely compressed as explained in the text.)

In Fig. 2 and Table 1 we show the defect structures that result from a mismatch of the (100) f.c.c. lattice parameter a (expressed in particle diameters). At small mismatches ($1.1 < a < 1.2$), the first few layers start out with a perfect square symmetry, but in subsequent layers the symmetry changes into hexagonally packed and randomly stacked layers through the gradual introduction of defects similar to Fig. 2A and B. At larger mismatch ($a \approx 1.2$) the template imposes a square symmetry in the first layer only in certain bands (Fig. 2C). Again, several layers higher, random stacked (111) planes are found everywhere. A similar structure is found for heteroepitaxial growth of CdTe on GaAs(100). Because of a large mismatch

(in one direction 14.6%) CdTe grows with (111) planes on the (100) template¹⁸. Of course, the interaction potential in this system is not hard-sphere-like, but the structural resemblances are striking. With still larger mismatch even the first layers is (defect-rich) hexagonal and the layers above stack again randomly, similar to Fig. 2D. For $a = \sqrt{2}$ the square lattice is, after rotation of 45° , commensurate with the original (100) plane with $a = 1$. The missing holes (Fig. 2E) in the first layer do not prevent the growth of a full f.c.c. crystal on the template. Around this lattice mismatch, the defect structures are similar to those found close to $a = 1$ (Table 1). It is interesting to note that at no lattice spacing was a body-centred (b.c.c.) crystal,

Table 1 Crystal structure formed on f.c.c. (100) plane as function of lattice spacing a

a	~ 1.0	$1.1-1.2$	~ 1.2	$1.2-1.3$	~ 1.3	$1.3-1.4$	$\sim \sqrt{2}$	$1.4-1.5$	~ 1.5	$1.5-2.0$
Symmetry of first layer	$\square$ (Fig. 1B, C)	$\square$	$\square/\triangleright$	$\triangleright$	$\diamond/\triangleright$ (Fig. 2C)	$\diamond$ (Fig. 2A)	$\diamond$ (Fig. 2E)	$\diamond$	$\diamond/\triangleright$	$\triangleright$ (Fig. 2D)
Symmetry of tenth layer	$\square$ (Fig. 1B, C)	$\triangleright$	$\triangleright$	$\triangleright$	$\triangleright$	$\triangleright$ (Fig. 2B)	$\diamond$	$\triangleright$	$\triangleright$	$\triangleright$

Here the lattice spacing a is given in multiples of the particle diameter. The symbols represent the symmetry of the crystalline layer: square ($\square$), hexagonal ($\triangleright$), 45° rotated square, see text ($\diamond$).

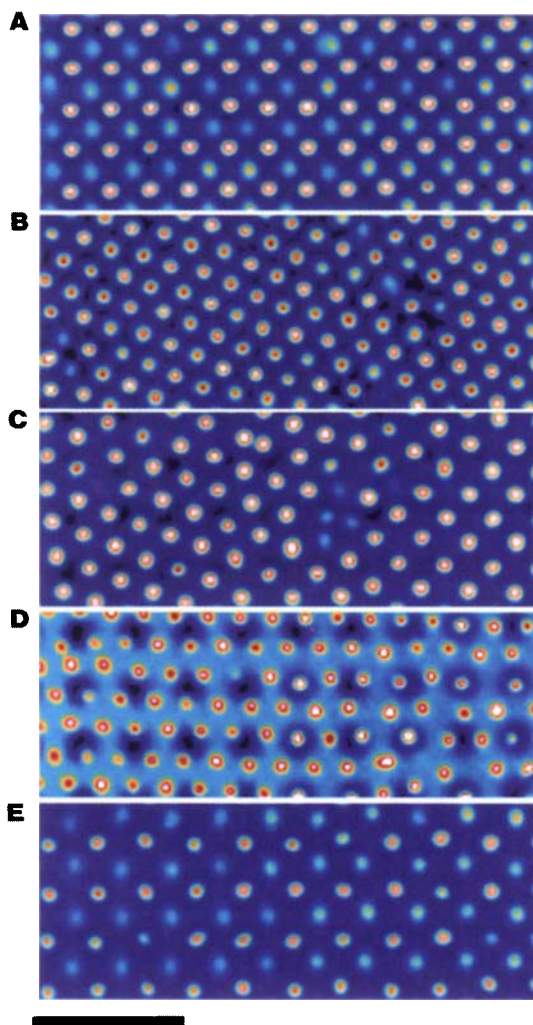


Figure 2 Local crystal plane symmetry of crystals grown on f.c.c. (100) planes with different lattice spacing a (given in multiples of the particle diameter). **A**, Part of optical section showing (100) symmetry of the first and the (first visible) second layer of spheres on a template with $a = 1.35$. **B**, As **A**, but ten layers above the template showing defect-rich hexagonal (111) symmetry. **C**, First layer above the template with $a = 1.3$ showing bands of (111) symmetry (left) and (100) symmetry (right). **D**, First layer of spheres with $a = 1.6$ showing a defect-rich hexagonal symmetry. **E**, First and second layers on a template with a close to 2 showing (100) symmetry, but rotated 45° compared to the crystal shown in Fig. 1. Scale bar, $5\text{ }\mu\text{m}$. The 'rainbow' false-colour table allows for easy visualization of which particle belongs in which layer and also makes it possible to recognize the template.

which has a (100) crystal plane with the same symmetry, formed. Probably, the difference in free energy of the less-dense b.c.c. crystal is too far off to be induced by the template.

Figure 3 gives another example of the manipulative possibilities of colloidal epitaxy. A simple change in the spacing between two adjacent (100) f.c.c. planes determines whether the (100) plane continues over the unpatterned region or whether a stack of (111) planes will form between the two f.c.c. crystals. Structures like these are important for the creation of optical wave guides.

Here we have shown that with colloidal epitaxy it is possible to create large hard-sphere f.c.c. single crystals oriented along crystal directions without twinning. It has also been shown that tailored crystals with different crystal orientations can be grown next to each other. Hard-sphere-like crystals are important for applications,

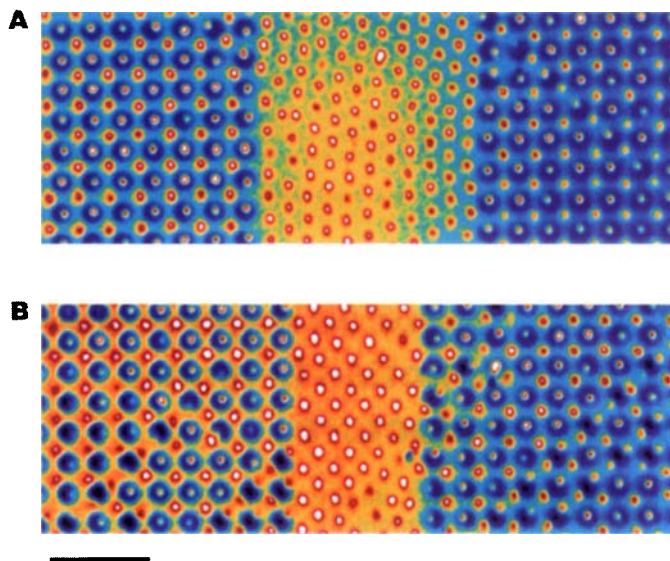


Figure 3 Confocal micrographs of the first layer of spheres on the (100) f.c.c. template with a close to 2 but with different distances between two templated regions (left, right): **A**, 11 sphere diameters: a hexagonal symmetry appears between the (100) faces. **B**, 7 sphere diameters: the square symmetry continues over the unpatterned polymer layer. Scale bar, $5\text{ }\mu\text{m}$.

because after slow drying these crystals can be turned into connected dielectric structures. In the case of particles with a silica coating this can be achieved through a mild sintering around 100°C which forms chemical siloxane bonds between the particles. Subsequently, the solvent can be changed without changing the dielectric structure.

Moreover, particles that interact with a hard-sphere-like potential provide the simplest model system with which to study quantitatively the defects that form after mismatching of the template lattice. Such mismatches can almost never be avoided in atomic hetero-epitaxy. There is no reason, however, why our technique could not be extended to charged spheres as well. In this case ionizable groups would have to be present on the template. Larger templates could be more easily created with other methods like photolithography or, as recently has been demonstrated with feature sizes of 25 nm thickness, with imprint lithography¹⁹. Further work is necessary to determine the influence of the sedimentation rate and volume-fraction on the epitaxial structures; preliminary experiments have already given indications of an interesting growth mechanism. For photonic bandgap materials it would be advantageous if the method could be extended to binary crystals as well. The use of a template could help systematic study of heterogeneous crystallization by observing the effects of the template under conditions at, or close to, homogeneous crystallization. □

Received 11 July; accepted 25 November 1996.

1. Flaugh, P. L., O'Donnell, S. E. & Asher, S. A. *Appl. Spectrosc.* **38**, 847–850 (1984).
2. Kamenetzky, E. A., Mangiocco, L. G. & Panzer, H. P. *Science* **263**, 207–210 (1994).
3. Sunkara, H. B., Jethmalani, J. M. & Ford, W. T. *Chem. Mater.* **6**, 362–364 (1994).
4. Chang, S., Liu, L. & Asher, S. A. *J. Am. Chem. Soc.* **116**, 6739–6744 (1994).
5. Vos, W. L. *et al. Phys. Rev. B* **53**, 16231–16235 (1996).
6. Tarhan, I. I. & Watson, G. H. *Phys. Rev. Lett.* **76**, 315–318 (1996).
7. Ackerson, B. J. & Schätzle, K. *Phys. Rev. E* **52**, 6448–6460 (1995).
8. Pusey, P. N. *et al. Phys. Rev. Lett.* **63**, 2753–2757 (1989).
9. Pusey, P. N. & van Megen, W. *Nature* **320**, 340–342 (1986).
10. Harland, J. L., Henderson, S. M., Underwood, S. M. & van Megen, W. *Phys. Rev. Lett.* **75**, 3572–3575 (1995).
11. Palberg, T., Mönch, W., Schwarz, J. & Leiderer, P. *J. Chem. Phys.* **102**, 5082–5087 (1995).
12. van Blaaderen, A. & Vrij, A. *Langmuir* **8**, 2921–2931 (1993).
13. Verhaegh, N. A. M. & van Blaaderen, A. *Langmuir* **10**, 1427–1438 (1994).
14. van Blaaderen, A. & Wiltzius, P. *Science* **270**, 1177–1179 (1995).
15. Parker, E. H. C. (ed.) *The Technology and Physics of Molecular Beam Epitaxy* (Plenum, New York, 1985).

16. Davis, K. E., Russel, W. B. & Glantsching, W. J. *Science* **245**, 507–510 (1989).
17. Frenkel, D. & Ladd, A. J. C. *J. Chem. Phys.* **81**, 318–3193 (1984).
18. Bourret, A., Fuoss, P., Geuillet, G. & Tatarenko, S. *Phys. Rev. Lett.* **70**, 311–314 (1993).
19. Chou, S. Y., Krauss, P. R. & Renstrom, P. J. *Science* **272**, 85–87 (1996).

Acknowledgements: We thank C. A. Murray for discussions and D. Frenkel for a critical reading of the manuscript. This work is part of the research programme of the Foundation for Fundamental Research of Matter (FOM) with financial support of the Netherlands Organization for Scientific Research (NWO).

Correspondence should be addressed to A.v.B. (e-mail: A.vanBlaaderen@chem.ruu.nl).

Current-induced organization of vortex motion in type-II superconductors

S. N. Gordeev*, P. A. J. de Groot*, M. Oussena*,
A. V. Volkov*, S. Pinfold*, R. Langan*, R. Gagnon†
& L. Taillefer†

* Physics Department, University of Southampton, Southampton SO17 1BJ, UK

† Physics Department, McGill University, Montréal (Québec), Canada H3A 2T8

When a magnetic field is applied to a type-II superconductor, it penetrates the sample in localized tubes of magnetic flux associated with quantized current vortices; under appropriate conditions, these vortices form an ordered lattice. In a material free of crystal defects, transport currents force this lattice to move and dissipate energy, giving the material a non-zero resistance. The presence of defects, however, can inhibit vortex motion, or even pin vortices to specific locations. Thus, to engineer materials with improved properties it is important to understand the motion of a driven vortex lattice in the presence of different kinds of pinning defects^{1,2}. Recent research has investigated vortex-lattice dynamics in the cases of weak, uniform pinning and strong but non-uniform pinning. Here we consider a different regime, in which the barriers to vortex motion at sample surfaces³ also play a crucial role. Our experiments on clean, detwinned $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$ crystals at temperatures around 80–90K reveal an interplay between surface pinning and weak bulk pinning that leads to the formation of a defect superstructure in the vortex lattice. This current-induced organization is similar to phenomena observed in the dynamics of sliding charge-density waves, and represents a fundamentally new kind of vortex dynamics.

Our experiments were performed on a high-quality, detwinned $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$ single crystal (1.1 mm $\times$ 0.35 mm $\times$ 65 μm). Information on crystal growth and detwinning can be found in ref. 4. Electrical contacts were made by painting onto the crystal surface four narrow strips of silver-epoxy which were subsequently cured at 450 °C in flowing oxygen. Magnetotransport measurements were performed using a sensitive SQUID picovoltmeter (resolution 5 pV) in magnetic fields up to 5 T applied along the crystalline *c*-axis and with the transport current applied along the *ab*-plane. The temperature stability was better than 2 mK.

Recent experiments² on NbSe_2 have shown that vortex dynamics are highly dependent on the value of the transport current. At low currents vortices flow non-uniformly, whereas at high currents a coherent motion of the whole vortex lattice is observed as a consequence of the current-induced decrease in the pinning potential. We have found that the vortex dynamics are also dependent on the type of transport current modulation. We have investigated the sample voltage for the same values of transport current in the cases of direct current (d.c.), and square-wave (s.w.) and asymmetrical square-wave (a.s.w.) currents (Fig. 1). All the curves shown here were obtained at a magnetic field $B = 2$ T and a temperature $T = 87$ K which is 2 K below the vortex lattice melting temperature, T_m . The d.c. data exhibit large noise. However, when using an s.w.

current of the same amplitude as the direct current, the voltage amplitude increases by a factor of up to 3, while the noise level decreases (Fig. 1a). We relate this difference to the effect of the surface barriers. According to Burlachkov *et al.*³, surface barriers should dominate in clean, untwinned $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$ single crystals at high temperature ($T > 80$ K). The flux flow in d.c. measurements is therefore governed by the rate at which the vortices pass through the surface barrier. Because this barrier is very sensitive to surface imperfections, this leads to highly non-uniform flux flow, with the possibility of channel formation. As a result voltage noise levels will be high. A different picture appears if the current changes polarity at a frequency, f_m , of the order of $10\text{--}10^2$ Hz (s.w.). In this case, the majority of the vortices never leave the sample, moving back and forth in an oscillating mode with a spatial amplitude of 0.4–4 μm . In this case the vortex motion is controlled mainly by weak bulk pinning—generally thought to be due to point defects such as oxygen vacancies—and hence the voltage measured will be higher than that for the direct current. Owing to the reduced effect of the surface pinning and possibly a smoothing of the inhomogeneous motion caused by the oscillatory nature of the vortex movement, the noise is lower in the s.w. case.

To investigate the interplay between surface and bulk pinning we devised a novel measurement technique. We applied an s.w. current with an asymmetry in the duration of the positive and negative parts of the square wave period (a.s.w. current). In this method the vortex system moves under the influence of large driving forces but with a low drift of vortices. Such measurements reveal a novel, previously unobserved type of flux flow. An a.s.w. current with a small asymmetry ($\sim 1\text{--}10\%$) induces a low-frequency ($\sim 10^{-2}$ Hz) modulation of the voltage amplitude (Fig. 1b). The measured voltage amplitude, V_{asw} , varies periodically, approximately between V_{sw} and V_{dc} . The amplitude and frequency of the observed oscillations are independent of the frequency of the applied current for $f_m > 40$ Hz. At lower values of f_m , the voltage amplitude variations cease to be periodic and become irregular, similar to the noise in measurements with a direct current. The oscillations of the type shown in Fig. 1b were observed over a substantial temperature range which could be as wide as 5 K, the upper temperature limit being close to, but always below, T_m .

We have found that, for low asymmetries, the period of the oscillations is related to the time needed for vortices to drift across the sample (the transit time τ_{tr}). The transit of vortices in an a.s.w.

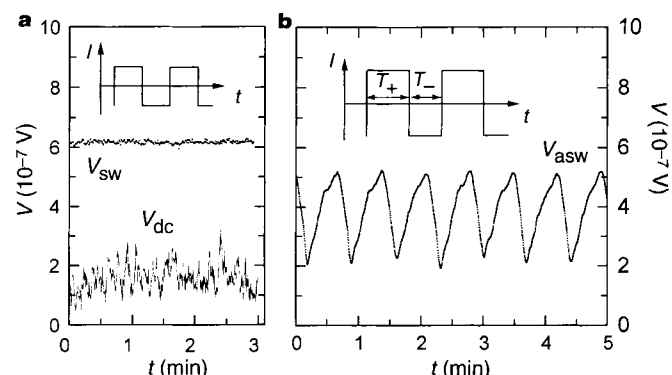


Figure 1 Voltage response of the $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$ crystal for different types of applied current modulation. A direct current (d.c.) induces a highly non-uniform vortex motion, a square-wave current (s.w.) results in a higher voltage amplitude with reduced noise, and an asymmetrical square-wave current (a.s.w.) reveals a low-frequency, periodic response. The amplitude of the transport current is identical for the three cases (3 mA). The insets show the time dependence of the current for s.w. and a.s.w. modulations. The frequency of the applied current, f_m , is 68 Hz (s.w., a.s.w.) and $\alpha = 0.1$ (a.s.w.). The timescale in the insets is expanded by a factor of 5,000 compared to that of the main figure.

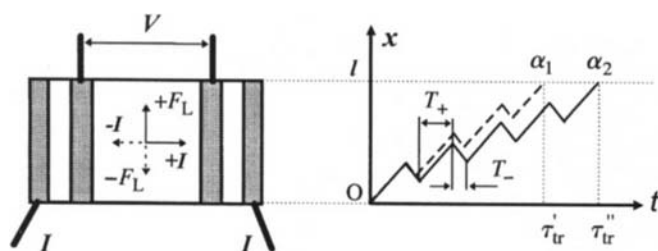


Figure 2 Left, schematic representation of the sample with current and voltage leads. The arrows show directions of the Lorentz force acting on a vortex for different polarities of the transport current. The magnetic field is normal to this figure. Right, plot showing schematically the motion of an individual vortex in the a.s.w. case for two different values for the asymmetry ($\alpha_1 > \alpha_2$). The time needed for the vortex to cross the sample (τ_{tr}) decreases with increasing α .

measurement is shown schematically in Fig. 2. According to Faraday's law of induction, the measured voltage is proportional to the velocity of the vortices, and hence the transit time can be estimated from the relation $\tau_{tr}^{-1} = \alpha V / Blh$, where l and h are the width of the sample and the distance separating the potential contacts, respectively (here $l = 0.35$ mm and $h = 0.78$ mm). V is the voltage averaged over a period of the oscillations (f_{osc}^{-1}). The factor α characterizes the asymmetry of the current square wave: $\alpha = (T_+ - T_-) / (T_+ + T_-)$, where T_+ and T_- are the duration of the positive and negative parts of the period respectively. The frequency of the oscillations is comparable to our estimate of τ_{tr}^{-1} ($\sim 10^{-2}$ Hz), is independent of f_m , and for $\alpha < 0.2$ increases linearly with voltage and asymmetry of modulation (Fig. 3) as predicted by the equation for τ_{tr}^{-1} . From these observations we conclude that: (1) the total vortex system drifts coherently across the sample without the formation of channels, and (2) the oscillations are directly related to a structural feature in the vortex lattice that moves across the sample together with the drifting vortices. There is some evidence from computer simulations⁵ that such an ordered defect superstructure may develop in the moving vortex lattice.

Recently D'Anna *et al.*⁶ observed peaks in the d.c. voltage noise spectrum indicative of coherent components to the vortex motion. Our conclusions are in contrast to their interpretation that the vortices move through channels which coherently open and close. The peak frequencies found in their studies are $\sim 10^3$ Hz (a factor 10^4 higher than our values for f_{osc}). Their measurements were made in the geometry $\mathbf{B} \perp \mathbf{c}$ for which the vortex lattice is highly anisotropic and hence a direct comparison with our results is difficult. To investigate the evolution of the coherent vortex motion observed in the a.s.w. case into the erratic d.c. vortex dynamics seen in our results, we have studied Fourier spectra of the $V(t)$ data for different values of α . The frequency of the oscillations is only proportional to τ_{tr}^{-1} for $\alpha < 0.2$ (Fig. 3). For $0.2 < \alpha < 0.4$ the linear dependence fails and the peaks in the spectra broaden. For $\alpha > 0.4$ as well as for direct currents ($\alpha = 1$) the Fourier spectrum did not show any clear peaks. In our studies the periodic reversal of the direction of the driving force is essential for establishing coherent oscillations. We only observed voltage oscillations for currents above a certain threshold value. From this we infer that the vortex lattice defect structure is organized through the influence of the driving force.

The existence of an organized defect structure in the vortex lattice for the a.s.w. measurements is supported by the results shown in the upper curve in Fig. 4. This represents the $V(t)$ dependence in an a.s.w. measurement for $\alpha = 0.1$. At $t = t_0$ the sign of the modulation asymmetry was changed to the opposite ($\alpha = -0.1$). This causes a reversal of the direction of the net vortex drift and therefore of the motion of the defect superstructure. The $V(t)$ curve displays

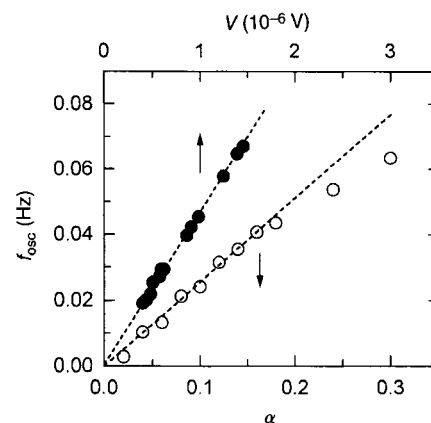


Figure 3 Frequency of the oscillations as function of the averaged voltage amplitude, V for $\alpha = 0.1$ (filled circles) and as function of α for $V = 4 \times 10^{-7}$ V (open circles) in the case of a.s.w. current.

the previously observed time dependence in reverse order: from $t = t_0$, it represents the mirror reflection of the last period immediately before the sign change.

Another feature of the observed organization is long-term 'memory' (Fig. 4, lower curve). The oscillations can be stopped at any time by interrupting the applied current. If, after a waiting time with zero current, the a.s.w. current is restored, the oscillations continue again with exactly the same phase and voltage readings as at the moment of interrupting the current. This effect is the result of the strong suppression of the pinning potential by the transport current. When the current is switched off the effective pinning barriers increase and all vortices are trapped by pinning centres near the places where they are at that moment in time. So the organized defect structure of the vortex lattice can be frozen in for long times (we have investigated waiting times up to 20 minutes). When the transport current is switched back on, the correlated motion of vortices continues.

The dynamics of complex systems under the influence of a driving force have been of considerable interest over the past few years. Computer simulations on simple models have revealed that

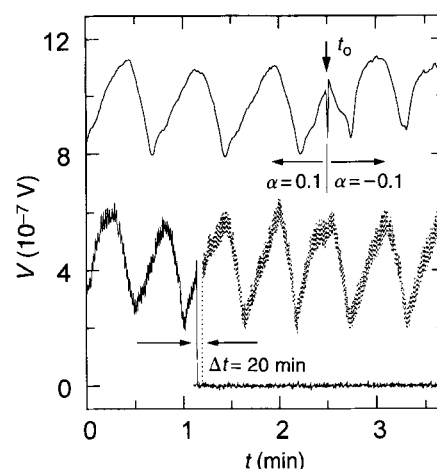


Figure 4 Upper curve; changing the sign of the asymmetry at $t = t_0$ reverses the net motion of the defect superstructure giving a mirror-like $V(t)$ dependence. The curve is for $I = 3$ mA and $\alpha = 0.1$ and is shifted along the vertical axis by 6×10^{-7} V. Lower curve; long-term memory effect. An interruption of the a.s.w. transport current 'freezes' the spatially organized distribution of vortices. The oscillations continue from the same phase when the current is restored. The applied current has the parameters $I = 3.5$ mA and $\alpha = 0.1$.

systems, for which chaotic dynamics are expected, may spontaneously develop organization under certain conditions⁷⁻⁹. Experiments on driven charge-density waves (CDWs) have given strong evidence for the occurrence of such organization¹⁰⁻¹². Electrical transport measurements on such systems have revealed strong noise for the sliding CDW which, for sufficiently large driving force (the voltage in the case of CDW), develops coherent components at well defined frequencies. The dynamics of CDWs also exhibit various memory effects. The system of weakly pinned vortices is very similar to the pinned CDW state and our findings demonstrate that organization of the dynamics can also occur for the driven vortex lattice. □

Received 29 April; accepted 26 November 1996.

- Jensen, H. J., Brass, A. & Berlinsky, A. J. *Phys. Rev. Lett.* **60**, 1676-1679 (1988).
- Yaron, U. *et al. Nature* **376**, 753-755 (1995).
- Burlachkov, L. *et al. Phys. Rev. B* **50**, 16770-16773 (1994).
- Gagnon, R., Lupien, C. & Taillefer, L. *Phys. Rev. B* **50**, 3458-3461 (1994).
- Braun, D. W. *et al. Phys. Rev. Lett.* **76**, 831-834 (1996).
- D'Anna, G. *et al. Phys. Rev. Lett.* **75**, 3521-3524 (1995).
- Sarkardei, M. R. & Jacobs, R. L. *Phys. Rev. E* **51**, 1929-1932 (1995).
- Tang, C., Wiesenfeld, K., Bak, P., Coppersmith, S. & Littlewood, P. *Phys. Rev. Lett.* **58**, 1161-1164 (1987).
- Coppersmith, S. N. & Littlewood, P. B. *Phys. Rev. B* **36**, 311-317 (1987).
- Fleming, R. M. & Grimes, C. C. *Phys. Rev. Lett.* **42**, 1423-1426 (1979).
- Dumas, J., Schlenker, C., Marus, J. & Buder, R. *Phys. Rev. Lett.* **50**, 757-760 (1983).
- Grüner, G. & Zettl, A. *Phys. Rep.* **119**, 117-232 (1985).

Acknowledgements: S.N.G. is on leave from the Moscow Institute of Radioengineering, Electronics and Automation. We thank H. J. Jensen and A. A. Zhukov for discussions. This work was supported by the UK Engineering and Physical Sciences Research Council. S.N.G. was supported by the Royal Society; L.T. was supported by the Canadian Institute for Advanced Research and the Sloan Foundation.

Correspondence and requests for materials should be addressed to P.A.J.d.G. (e-mail: pajdeg@phys.soton.ac.uk).

Spreading-rate dependence of the extent of mantle melting beneath ocean ridges

Yaoling Niu* & Roger Hékinian†

* Department of Earth Sciences, The University of Queensland, Brisbane, Queensland 4072, Australia

† Department of Marine Geosciences, IFREMER, Centre de Brest, BP37, 29287 Plouzané, France

Abyssal peridotites and mid-ocean-ridge basalts (MORBs) are complementary products of the mantle melting and melt-extraction processes that create the ocean crust. Studies of abyssal peridotites¹⁻⁴ and MORBs² have showed that the extent of mantle melting is high beneath hotspot-influenced shallow ridges, and is low beneath deep ridges away from hotspots. These results have led to the recognition of a global correlation of MORB composition with ridge depth⁵, and to the notion that mantle temperature variation exerts the primary control on the extent of melting beneath ocean ridges^{2,5-8}. This conclusion is, however, based largely on data from slow-spreading ridges in the Atlantic and Indian oceans. At the fast-spreading East Pacific Rise (EPR), there is little correlation between MORB chemistry and ridge depth^{9,10}, an observation that has proved puzzling⁸⁻¹². Here we show that abyssal peridotites from the EPR¹³⁻²¹ are extremely depleted in basaltic major-element components—significantly more so than peridotites from ridges away from hotspots in the Atlantic and Indian oceans—indicating that the EPR peridotites are residues of the highest extents of melting. These abyssal peridotite data and existing MORB major-element data¹² both suggest that the extent of mantle melting beneath normal ocean ridges increases with increasing spreading rate.

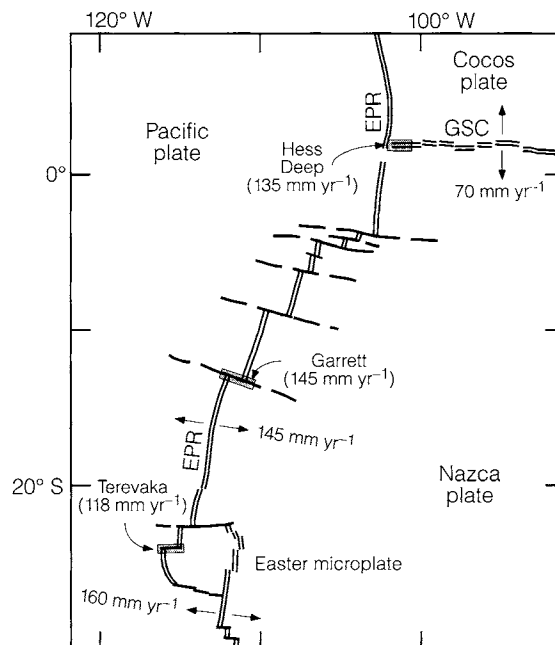


Figure 1 Schematic map of the East Pacific Rise (EPR) axis and the vicinity showing the Hess Deep, Garrett transform and Terevaka transform which are the only locations at the EPR where abyssal peridotites have been recovered¹³⁻²¹. GSC is the Galapagos spreading centre. Numbers with mm yr⁻¹ are full spreading rates.

There had been no detailed sampling of abyssal peridotites from the EPR until very recent investigations at three localities: Hess Deep¹³⁻¹⁵, Garrett transform¹⁶⁻¹⁸ and Terevaka transform¹⁹⁻²¹ (Fig. 1). We found that these EPR peridotites are highly depleted in harzburgites with little (≤ 1 vol.%) clinopyroxene, as found in drilled samples at Hess Deep¹⁵. Residual minerals in these peridotites plot at the most depleted end of the array defined by abyssal peridotite samples from slow-spreading ridges in the Atlantic and Indian oceans (Fig. 2). It has been demonstrated that Cr# ($= \text{Cr}/[\text{Cr} + \text{Al}]$) in spinel and Al_2O_3 contents in orthopyroxene and clinopyroxene of residual peridotites are sensitive indicators of the extent of melting^{1-3,22,23} because Al, a moderately incompatible element during mantle melting, is progressively depleted in these phases during melting. Hence, Fig. 2 shows that the extent of melting beneath the fast-spreading ($> 110 \text{ mm yr}^{-1}$) EPR is higher than beneath these slow-spreading ($\leq 25 \text{ mm yr}^{-1}$) ridges. Note that this difference in relative extent of melting between fast- and slow-spreading ridges is remarkable when data from hotspot-influenced ridges are excluded. The difference could be ascribed to a less-depleted fertile mantle beneath these slow-spreading ridges than beneath the EPR, but this would be fortuitous.

Although we do not have peridotite data from intermediate-spreading ridges at present, the available data do suggest that the distinct difference in extent of melting may be related to spreading-rate differences. Although abyssal peridotite data are available only from three locations at the EPR, they probably represent the average characteristics of melting residues beneath the EPR, at least in the equatorial and southern region (Fig. 1) because ridges in this broad region, as in other portions of the EPR, have normal depth variation, topography and morphology thermally unaffected by any known hotspots, and erupt MORB that are typical of the EPR^{24,25}. Alternatively, the high extents of melting shown by the EPR peridotites could be due to a hotter EPR mantle. However, Zhang and Tanimoto²⁶ showed that the deeper bound of seismic low-velocity anomalies associated with ocean ridges is $\sim 100 \text{ km}$ deep, and is independent of spreading rate. Whereas Su *et al.*²⁷

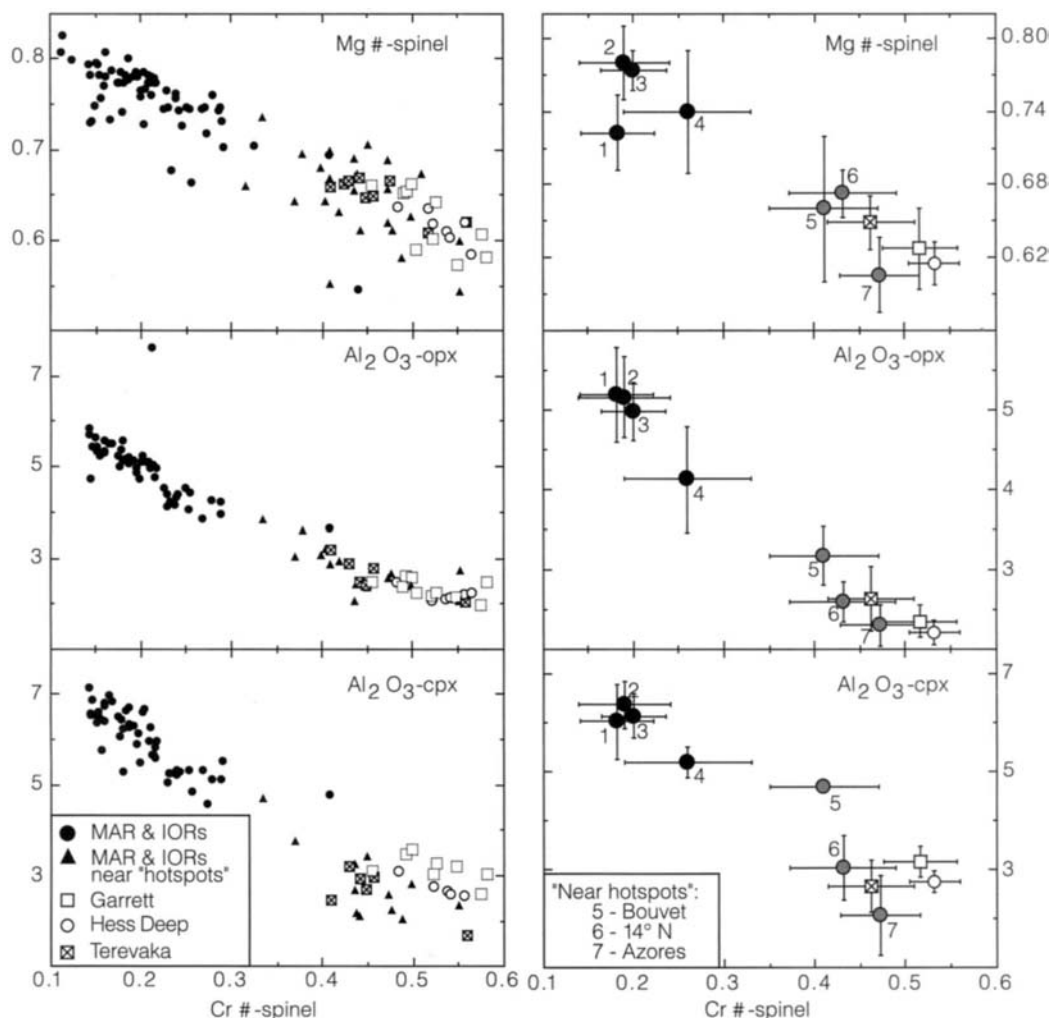


Figure 2 Plots of Cr# ($\text{Cr}/(\text{Cr} + \text{Al})$) and Mg# ($\text{Mg}/(\text{Mg} + \text{Fe}^{2+})$) in residual spinels and Al_2O_3 contents in residual clinopyroxene (cpx) and orthopyroxene (opx) to show that EPR peridotites plot at the most depleted end of the array defined by samples from slow-spreading ridges in the Atlantic and Indian Oceans. Left panels are the raw data. Right panels are location averages: 1, Atlantis II fracture zone; 2, Vulcan fracture zone; 3, Islas Orcadas fracture zone; 4, Bullard fracture

zone; 5, Bouvet fracture zone (near Bouvet hotspot) at the Indian Ocean ridges (IORs); 6, 15° 37' N axial valley (near 14° N, an incipient mantle plume⁴²); and 7, 43° N fracture zone (near the Azores hotspot) at the Mid-Atlantic Ridge (MAR). The data are from refs 1–4, 20, 43–47, and this study. Spreading rates are $\leq 25 \text{ mm yr}^{-1}$ for sample locations in the Atlantic and Indian Oceans (1–7), and $\geq 110 \text{ mm yr}^{-1}$ for sample locations in the Pacific Ocean⁴⁶.

argued for a deep ($\geq 300 \text{ km}$ beneath all ridges) origin of ocean-ridge seismic-velocity anomalies, work using higher-frequency surface waves suggests²⁸ that velocity variations beneath ocean ridges are confined to the uppermost 100 km. This suggests that mantle potential temperature variation may not be significant beneath normal ridges unaffected by hotspots²⁹.

Given the fact that mantle melting beneath ocean ridges is caused by decompression as mantle adiabatically upwells, and that mantle upwelling results from plate separation, fast plate separation at the EPR may be responsible for the high extents of melting evident from the EPR peridotites. If so, it should be evident in MORB chemistry that the extent of mantle melting increases with increasing spreading rate. Indeed, the averaged Al_8 (that is, Al_2O_3 wgt% corrected for crystal fractionation effect to 8.0% MgO ; refs 5, 12) and Ca_8/Al_8 in MORB correlate significantly with spreading rate (Fig. 3). Because Al is a moderately incompatible element, its abundance in partial melts decreases while the Ca/Al ratio increases with increasing melting^{7,22}. Therefore, correlations in Fig. 3 show that the extent of melting beneath ocean ridges increases with increasing spreading rate. In Fig. 3a we plot all the data of ref. 12 averaged within every 15 mm yr^{-1} spreading rate interval. Figure 3b shows the data

averaged within every 20 mm yr^{-1} interval excluding samples from hotspot-influenced ridges and samples with $\text{K}/\text{Ti} > 0.3$ (due to anomalously enriched sources) from all ridges. Apparently, the correlations in Fig. 3b are significantly improved when thermal anomalies associated with hotspots and the effect of first-order source compositional variations are removed. Note that the correlations in Fig. 3b are better described by curves (power functions) than by straight lines, consistent with model predictions^{30,31}.

Whereas various differences exist in MORB chemistry between slow- and fast-spreading ridges^{7–12,32}, previous studies have all concluded that the extent of melting is independent of spreading rate, except at very slow-spreading rates ($< 20 \text{ mm yr}^{-1}$)^{29,33}, primarily because of the use of Na. There are two apparent difficulties using Na as an indicator of the extent of melting. First, Na is an incompatible minor element, which is known to suffer from fertile mantle heterogeneity²⁹. Although abundances of Ca and Al may not be uniform in the mantle, their variability should be relatively small as these elements are major (several per cent) components of mantle material, and their behaviour is largely governed by phase equilibria both under subolidus conditions and during melting^{1,22,34}. Second, due to its very incompatible behaviour, Na tends to enter the melt at

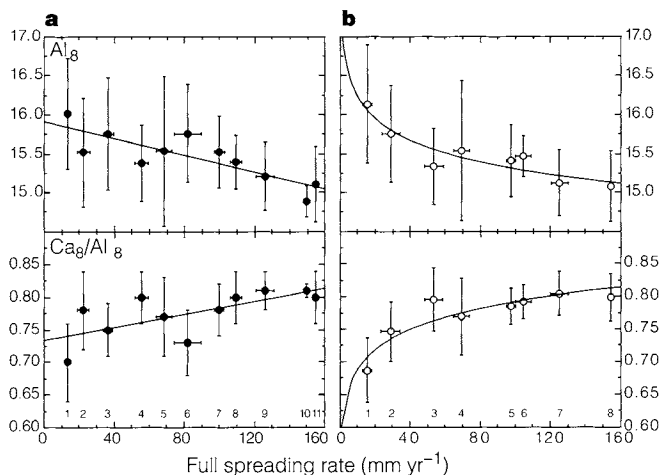


Figure 3 Plots of Al_8 and Ca_8/Al_8 in global MORB averaged by spreading rate against full spreading rate; these statistically significant correlations indicate that the extent of melting increases with increasing spreading rate. The global MORB data base and correction procedure are described in ref. 12. The spreading rate data are from ref. 48. The averaging is done for both spreading rate and Al_8 and Ca_8/Al_8 within a given spreading rate window. The bars are ± 1 standard deviation. **a**, All the data averaged within every 15 $mm\ yr^{-1}$: 1, spreading rate (SR) < 15 $mm\ yr^{-1}$, number of samples (N) = 79; 2, SR = 15–30, N = 821; 3, SR = 30–45, N = 204; 4, SR = 45–60, N = 350; 5, SR = 60–75, N = 121; 6, SR = 75–90, N = 17; 7, SR = 90–105, N = 347; 8, SR = 105–120, N = 89; 9, SR = 120–135, N = 6; 10, SR = 135–150, N = 29; 11, SR > 150, N = 239. The correlation coefficients are $R = -0.829$ for Al_8 and $R = 0.685$ for Ca_8/Al_8 . These R values are significant statistically at > 99% and 95% confidence levels respectively. **b**, Data averaged within every 20 $mm\ yr^{-1}$ after excluding samples from hotspot-influenced ridges/ridge segments (that is, the Iceland, Azores, Galapagos, Juan de Fuca) and samples with $K/Ti > 0.3$: 1, SR < 20, N = 89; 2, SR = 20–40, N = 431; 3, SR = 40–60, N = 300; 4, SR = 60–80, N = 106; 5, SR = 80–100, N = 176; 6, SR = 100–120, N = 252; 7, SR = 120–140, N = 5; and 8, SR > 140, N = 235. The linear correlation coefficients are $R = -0.877$ (at < 99% confidence levels) for Al_8 and $R = 0.791$ (at < 98% confidence levels) for Ca_8/Al_8 . The systematics as a function of spreading rate are, however, better described by simple power functions: $Y = 17.241 X^{-0.026}$ ($R = -0.928$) for Al_8 and $Y = 0.579 X^{0.061}$ ($R = 0.912$) for Ca_8/Al_8 .

very early stages of melting. Na abundances in the melt thus decrease rapidly with melting at low extents, but slowly with progressive melting as the result of dilution. Therefore, Na is insensitive to extensive melting, particularly when the effect of source heterogeneity cannot be properly corrected for. In contrast, Ca increases and Al decreases more gradually in the melt with melting. In particular, the Ca/Al ratio increases nearly linearly with increasing melting until clinopyroxene is exhausted at very high extents of melting ($\geq 25\%$)^{7,22}. Therefore, Al and Ca/Al are effective measures of the extent of melting for the entire melting range beneath ridges. The averaged Na_8 is found (data not shown) to exhibit no correlation (such as in Fig. 3a) or a weak correlation (such as in Fig. 3b; dominated by two points in the < 40 $mm\ yr^{-1}$ range) with spreading rate, which is not surprising given the above reasons. The large variation range (± 1 standard deviation) in Al_8 and Ca_8/Al_8 for a given spreading-rate window (Fig. 3b) reflects the combined effect of mantle source heterogeneity with respect to these elements, detailed differences in melting processes, and imperfect corrections for crystal fractionation, but the systematics defined by averaged data (averaging out these various factors) as a function of spreading rate is evident.

We propose that the observed variations in extent of melting with spreading rate result from variations in final depth of melting²⁹ (Fig. 4).

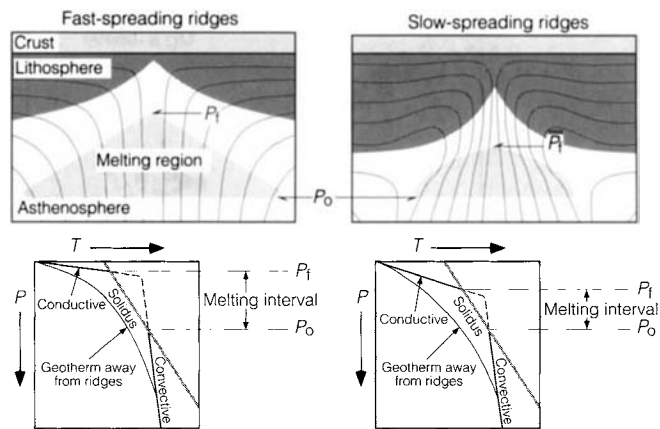


Figure 4 Cartoon comparing uppermost mantle thermal structures between fast- and slow-spreading ridges. Even if the initial melting depth is the same (that is, the same mantle potential temperature), the thermal structure at shallow levels is largely determined by conductive cooling to the surface^{30,31,35}. An upwelling mantle along the adiabat (convective thermal gradient) begins to melt when intersecting the solidus at P_0 . Decompression melting continues as the melting mantle upwells until conductive cooling to the surface dominates over the adiabat at a shallow level P_i . Fast upwelling beneath fast-spreading ridges allows the adiabat to extend to a shallower level against conductive cooling than slow upwelling beneath slow-spreading ridges. Therefore, below fast-spreading ridges (for example, the EPR), decompression melting continues up to a shallower level, the melting interval between initial (P_0) and final (P_i) depth of melting is larger, and more melt is produced from a given parcel of mantle than beneath slow-spreading ridges (for example, ridges in the Atlantic and Indian oceans). The implication is that the average crust should be thicker in the Pacific than in the Atlantic and Indian oceans. The flowlines are modified from ref. 49.

As mantle upwelling beneath ocean ridges results from plate separation, mantle upwelling rate is proportional to spreading rate^{30,31,35}. Given the fact that mantle temperature profile beneath a ridge is determined by both convective (adiabat) and conductive thermal gradients^{29–31}, fast upwelling beneath fast-spreading ridges allows the adiabat to extend to a shallower level against conductive cooling to the surface. By contrast, with slower upwelling beneath slow-spreading ridges, conductive cooling to the surface extends to a greater depth against the adiabat (Fig. 4). Consequently, decompression melting continues up to a shallower level and more melt is produced from a given parcel of mantle beneath fast-spreading ridges than beneath slow-spreading ridges^{30,31,36}. Although this concept has long been established^{30,36}, its effect has not been seriously considered in models^{5,6,8} of MORB genesis. Niu and Batiza⁷ showed that mantle melting beneath ocean ridges stops at levels significantly deeper than the Moho because MORB chemistry is inconsistent with melting to the Moho. Shen and Forsyth²⁹ stressed that variation in final depth of melting due to conductive cooling is important in affecting MORB chemistry. Also, Zhang and Tanimoto²⁶ showed that at a shallow level (for example, at 36 km) beneath ocean ridges, S-wave low-velocity anomalies correlate strongly with spreading rate, indicating a stronger influence of conductive cooling beneath slow-spreading ridges than beneath fast-spreading ridges at this level. Asimow *et al.*³⁷ argued that the effect of pressure-induced solid–solid phase transitions (that is, garnet–spinel and spinel–plagioclase stability field transitions) is to suppress melting, and may potentially be the cause of melting cessation beneath ocean ridges. Our new observations (Figs 2 and 3), model predictions^{30,31,36} and mantle tomographic studies²⁶, taken together, argue persuasively that the final depth of melting is

primarily determined by the conductive cooling to the surface (Fig. 4). Although a precise determination of the final depth of melting as a function of spreading rate requires more observational data²⁹, there is the suggestion from ref. 26 that melting may stop at depths ≥ 30 km beneath very slow-spreading ridges, but at depths perhaps significantly less than 30 km beneath fast-spreading ridges.

Compared with melting residues produced by peridotite melting experiments²², the highly depleted residual mineral compositions and the extent of depletion to which clinopyroxene is exhausted in the EPR peridotites indicate that these peridotites are residues of $\geq 25\%$ melting. This high value in extent of melting is significantly higher than 10%, a value that has been advocated to be typical of mantle melting beneath global ocean ridges^{38,39}. Calculations⁷ show that the range of averaged Al_8 and Ca_8/Al_8 values (Fig. 3) corresponds to $\sim 10\%$ melting at the slow-spreading rate end and $\sim 22\%$ melting at the fast-spreading rate end of the entire spreading rate variation range. Thus, abyssal peridotites and MORB both indicate $\geq 20\%$ melting beneath the EPR. This suggests that there is no need to invoke long-distance (>60 km) lateral melt migration to the ridge axis required in models that assume 10% melting to explain the crustal accretion at the very narrow axial zone at the EPR (see reviews in refs 31, 35).

Another important implication is that oceanic crust should be, on average, thicker in the Pacific than in the Atlantic and Indian oceans, which is in accord with model predictions that crustal thickness increases with spreading rate^{30,31,36}. Although this seems to be inconsistent with the notion (based on seismic velocity data) that oceanic crustal thickness is generally constant (~ 6 – 7 km) and is independent of plate spreading rate^{33,40}, thin crust and peridotite outcrops have been observed within axial zones at slow-spreading ridges⁴¹ but not at fast-spreading ridges. Therefore, crustal thickness derived from seismic data must be used with caution. Relatively few ridges or ridge segments are thermally affected by hotspots. Exclusion of these few anomalies from ridge petrology data sets leads to a clear suggestion that spreading-rate variation is the primary variable that determines both the extent of mantle melting beneath ocean ridges and MORB chemistry. $\square$

Received 25 June; accepted 20 November 1996.

- Dick, H. J. B. & Fisher, R. L. in *Proc. 3rd Int. Kimberlite Conf/Vol. II* (ed. Kompobst, I.) 295–308 (New York, 1984).
- Dick, H. J. B., Fisher, R. L. & Bryan, W. B. *Earth Planet. Sci. Lett.* **69**, 88–106 (1984).
- Michael, P. J. & Bonatti, E. *Earth Planet. Sci. Lett.* **73**, 91–104 (1985).
- Johnson, K. T. M., Dick, H. J. B. & Shimizu, N. *J. Geophys. Res.* **95**, 2661–2678 (1994).
- Klein, E. M. & Langmuir, C. H. *J. Geophys. Res.* **92**, 8089–8115 (1987).
- McKenzie, D. & Bickle, M. J. *J. Petrol.* **29**, 625–679 (1988).
- Niu, Y. & Batiza, R. *J. Geophys. Res.* **96**, 21753–21777 (1991).
- Langmuir, C. H., Klein, E. M. & Plank, T. in *Mantle Flow and Melt Generation at Mid-ocean Ridges* (eds Phipps Morgan, J., Blackman, D. K. & Sinton, J. M.) 183–280 (AGU Monogr. 71, Am. Geophys. Union, Washington DC, 1992).
- Brodholt, J. P. & Batiza, R. *J. Geophys. Res.* **94**, 4231–4239 (1989).
- Klein, E. M. & Langmuir, C. H. *J. Geophys. Res.* **94**, 4241–4252 (1989).
- Batiza, R., Hékinian, R., Bideau, D. & Francheteau, J. *Geophys. Res. Lett.* **22**, 3067–3070 (1995).
- Niu, Y. & Batiza, R. *J. Geophys. Res.* **98**, 7887–7902 (1993).
- Francheteau, J. *et al. Earth Planet. Sci. Lett.* **101**, 282–295 (1990).
- Hékinian, R. *et al. J. Geophys. Res.* **98**, 8069–8094 (1993).
- Dick, H. J. B. & Natland, J. H. *Proc. ODP Sci. Res.* **147**, 103–134 (1996).
- Hékinian, R., Bideau, D., Cannat, M., Francheteau, J. & Hébert, R. *Earth Planet. Sci. Lett.* **108**, 259–273 (1992).
- Hékinian, R., Bideau, D., Hébert, R. & Niu, Y. *J. Geophys. Res.* **100**, 10163–10185 (1995).
- Constantin, M., Hékinian, R. & Hébert, R. *Geology* (in the press).
- Francheteau, J. *et al. (abstr.) Eos* **75**, 582 (1994).
- Constantin, M., Hékinian, R., Ackermann, D. & Stoffers, P. in *Mantle and Lower Crust Exposed in Oceanic Ridges and Ophiolites* (eds Visser, R. L. M. & Nicolas, A.) 71–120 (Kluwer, Dordrecht, 1995).
- Hékinian, R. *et al. J. Volcanol. Geotherm. Res.* (in the press).
- Jaques, A. L. & Green, D. H. *Contrib. Mineral. Petrol.* **73**, 287–310 (1980).
- Dick, H. J. B. & Bullen, T. B. *Contrib. Mineral. Petrol.* **86**, 54–76 (1984).
- Langmuir, C. H., Bender, J. F. & Batiza, R. *Nature* **332**, 422–429 (1986).
- Sinton, J. M., Smaglik, S. M., Mahoney, J. J. & Macdonald, K. C. *J. Geophys. Res.* **96**, 6133–6155 (1991).
- Zhang, Y.-S. & Tanimoto, T. *Nature* **355**, 45–49 (1992).
- Su, W.-J., Woodward, R. L. & Dziewonski, A. M. *Nature* **360**, 149–152 (1992).
- Phipps Morgan, J., Morgan, J. W., Zhang, Y.-S. & Smith, W. H. F. *J. Geophys. Res.* **100**, 12753–12767 (1995).
- Shen, Y. & Forsyth, D. W. *J. Geophys. Res.* **100**, 2211–2237 (1995).
- Reid, I. & Jackson, H. R. *Mar. Geophys. Res.* **5**, 165–172 (1981).
- Forsyth, D. W. in *Mantle Flow and Melt Generation at Mid-Ocean Ridges* (eds Phipps Morgan, J., Blackman, D. K. & Sinton, J. M.) 1–66 (AGU monogr. 71, Am. Geophys. Union, Washington DC, 1992).

- Nisbett, E. G. & Pearce, J. A. *Nature* **246**, 468–469 (1973).
- Bown, J. W. & White, R. S. *Earth Planet. Sci. Lett.* **121**, 435–449 (1994).
- Dick, H. J. B. *Am. J. Sci.* **277**, 801–832 (1972).
- Turcotte, D. L. & Phipps Morgan, J. in *Mantle Flow and Melt Generation at Mid-ocean ridges* (eds Phipps Morgan, J., Blackman, D. K. & Sinton, J. M.) 155–182 (AGU monogr. 71, Am. Geophys. Union, Washington DC, 1992).
- Bottinga, Y. & Allègre, C. *Phil. Trans. R. Soc. Lond. A* **288**, 501–525 (1978).
- Asimow, P. D., Hirschmann, M. M., Ghiorso, M. S., O'Hara, M. J. & Stolper, E. *Geochim. Cosmochim. Acta* **59**, 4489–4506 (1995).
- Klein, E. M., Plank, T. & Langmuir, C. H. *RIDGE Events* **2**, 11–12 (1991).
- Forsyth, D. W. *J. Geophys. Res.* **98**, 16073–16079 (1993).
- Chen, Y. J. *Geophys. Res. Lett.* **19**, 753–756 (1992).
- Cannat, M. *J. Geophys. Res.* **101**, 2847–2857 (1996).
- Bourgault, H. *et al. Earth Planet. Sci. Lett.* **88**, 27–36 (1988).
- Shibata, T. & Thompson, G. *Contrib. Mineral. Petrol.* **93**, 144–159 (1986).
- Dick, H. J. B. *Geol. Soc. Special Publ.* **42**, 71–105 (1989).
- Johnson, K. T. M. & Dick, H. J. B. *J. Geophys. Res.* **97**, 9219–9241 (1992).
- Cannat, M., Bideau, D. & Bourgault, H. *Earth Planet. Sci. Lett.* **109**, 87–106 (1992).
- Hébert, R., Bideau, D. & Hékinian, R. *Earth Planet. Sci. Lett.* **65**, 107–125 (1983).
- DeMets, C., Gordon, R. G., Argus, D. F. & Stein, S. *Geophys. J. Int.* **101**, 425–478 (1990).
- Scott, D. R. & Stevenson, D. J. *J. Geophys. Res.* **94**, 2973–2988 (1989).

Acknowledgements: We thank M. Bohn for assistance with mineral microprobe analysis (SX 50 Camebax, IFREMER); and H. Dick and J. Sinton for reviews. The Pacific peridotites were collected during 1990, 1991 and 1993 with the submersible *Nautil* and its support ship *Nadir*. The dives were sponsored by IFREMER and INSU and organized by IFREMER-GM and UBO (Chief Scientists: J. Francheteau for PITO and Hess Deep cruises, and R. Hékinian for Garrett cruise). This work was supported by the Bilateral Science and Technology Program of Australia and the Ministère de l'Éducation de l'enseignement Supérieur et de la Recherche de France.

Correspondence and requests for materials should be addressed to Y.N. (e-mail: niu@earthsciences.uq.edu.au).

Corrugated slip surfaces formed at ridge-transform intersections on the Mid-Atlantic Ridge

J. R. Cann*, D. K. Blackman*, D. K. Smith†, E. McAllister*, B. Janssen*, S. Mello*, E. Avgerinos*, A. R. Pascoe* & J. Escartin†

* Department of Earth Sciences, University of Leeds, Leeds LS2 9JT, UK

† Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA

The strips of ocean crust formed at the inside corners of both transform and non-transform offsets on the Mid-Atlantic Ridge are punctuated by topographic highs—the 'inside-corner highs'^{1–3}—where plutonic rocks (including gabbros and peridotites) are frequently found^{4,5}. Current tectonic models consider the inside-corner highs to be lower-crust and upper-mantle materials that have been exhumed by low-angle detachment faults dipping away from the inside corner to beneath the ridge axis^{3,6–8}. But much of the evidence for the existence of such faults has hitherto been circumstantial. Here we present sonar images of two ridge-transform intersections on the Mid-Atlantic Ridge (near 30°N), which show that both active and 'fossil' inside-corner highs are capped by planar, dipping surfaces marked by corrugations and striations oriented parallel to the plate spreading direction. Although these surfaces may be the low-angle detachment faults envisaged by the models, they dip at much shallower angles than expected. This could be explained by the lubricating presence of serpentinized peridotite, fragments of which have been dredged from both surfaces. Alternatively, these slip surfaces may instead represent failure surfaces in serpentine-lubricated landslide zones.

Sea-floor morphology in the vicinity of the Atlantis transform fault (Fig. 1) is typical of that near many large transform faults that offset the Mid-Atlantic Ridge. Two strips of anomalous crust ~ 15 km wide run on either side of the fault trace from the inside corners of the ridge-transform intersections (RTIs) parallel to the

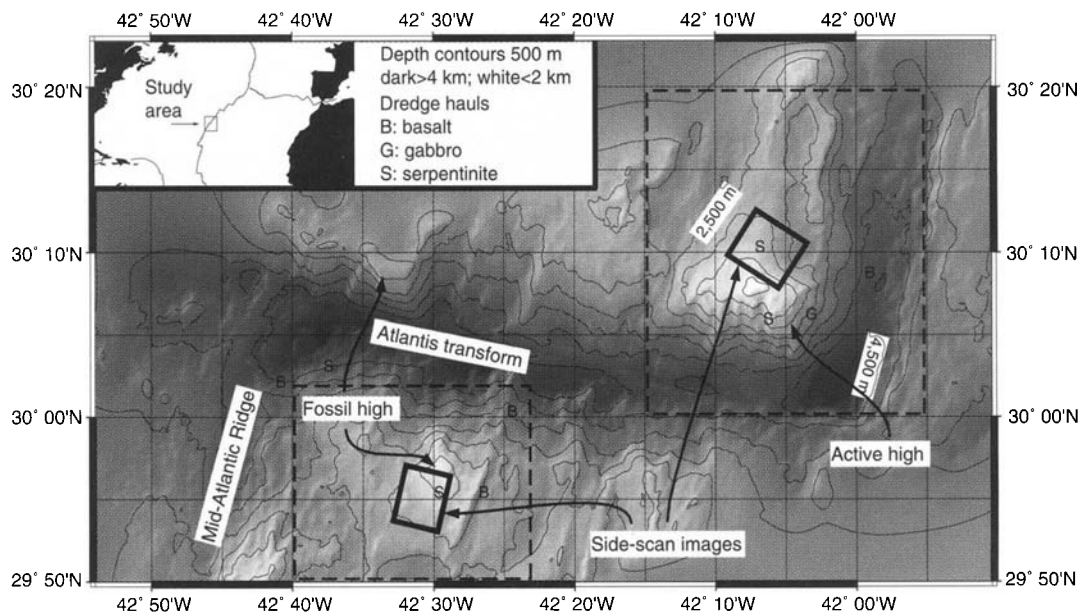


Figure 1 Map of the Atlantis transform and adjacent ridge segments, showing bathymetry, illuminated sea-floor relief, rock sample locations and active and fossil inside-corner highs. Bathymetry was collected on Cruise 100 of RRS *Charles Darwin* in Spring 1996, and coverage is complete within the illuminated region. Tracks ran NE-SW at an angle both to the ridge axes and the transform fault and were navigated by GPS; weather was good during the survey. Depths

are gridded at 100-m intervals for this image and were measured with a Simrad EM12 multibeam bathymetry system. Contours are at 500-m intervals, and depths range from over 5,500 m to less than 1,000 m. Letters show locations and rock types in dredge hauls from both this and an earlier cruise^{17,18}. Large dashed boxes outline the areas of Fig. 2a (eastern) and 2b (western); smaller bold boxes show the areas of Fig. 3a (eastern) and 3b (western).

transform fault. Instead of the normal mid-ocean-ridge morphology of volcanic constructional features cut by faults running parallel to the spreading centre^{9,10}, the strips show an irregular morphology interrupted by blocky topographic highs. These inside-corner highs rise 1,000 m or more above the surrounding sea floor, and appear to be generated at the inside corners and then spread away parallel to the transform fault¹⁻³. At RTIs where inside-corner highs are actively being constructed, the topographic relief is high and slopes are steep. The alternation in topography between inside-corner highs and intervening topography has been interpreted to reflect episodicity in style of crustal accretion through time¹¹⁻¹⁴.

The two RTIs at each end of the Atlantis transform (Fig. 1) appear to be in different stages of tectonic evolution: the inside corner at the western intersection of the Mid-Atlantic Ridge with the Atlantis transform fault lacks an inside-corner high and contains irregular hills and valleys with an amplitude of a few hundred metres; the eastern inside corner has a topographic high rising steeply from the RTI to a depth of less than 1,000 m, which appears to be actively forming. Our new data cover this active high, as well as one inside-corner high on the south side of the transform (created at the

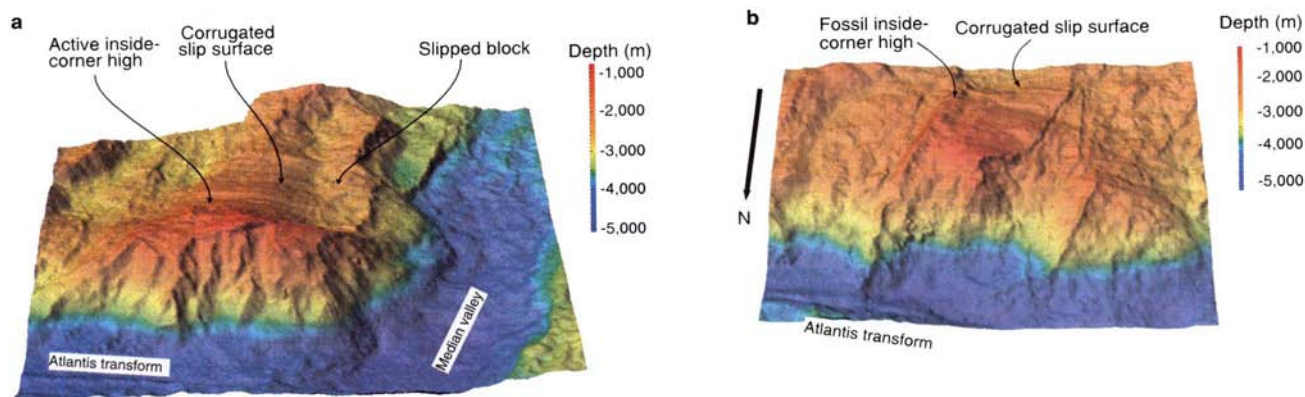


Figure 2. a, Three-dimensional shaded relief image of the bathymetry of the active inside-corner high at the eastern ridge-transform intersection, viewed from the south, and illuminated from the NW. The area of this figure is outlined by the eastern large dashed box on Fig. 1, and is 30 km from east to west, and 35 km from north to south. The positions of features mentioned in the text are shown. Note the corrugated surface curving from sub-horizontal to dip at $\sim 10^\circ$ towards the spreading axis, and the slipped block lying on top of the corrugated surface. Side-scan sonar images show that this block has volcanic morphology on its top

surface. Simrad bathymetry data was gridded at 100-m intervals for this image. **b**, Similar shaded relief image of the bathymetry of the fossil high south of the transform fault. The area of this figure is outlined by the western dashed box on Fig. 1, and is 20 km square. The nearly horizontal corrugated surface is ~ 10 km square in the centre of the image. Both corrugated surfaces are terminated at the end furthest from the spreading axis by normal faults throwing down away from the axis.

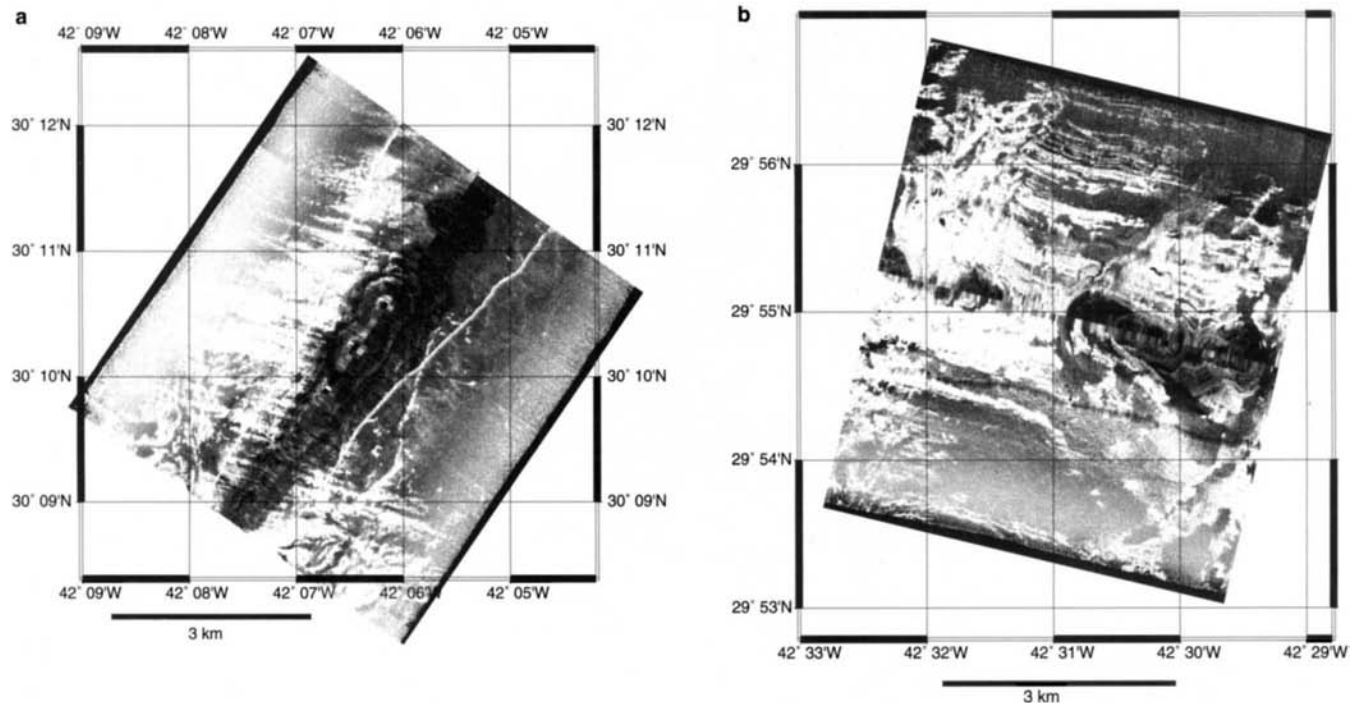


Figure 3 Side-scan sonar images of the striations on the corrugated surfaces at active (a) and fossil (b) inside-corner highs. Location of panels is shown by the smaller boxes in Fig. 1. Images were generated by the Tobi deep-towed side-scan sonar instrument, operating at 30/32 kHz, with a swath width of 6 km and a horizontal resolution of ~ 10 m. Lighter areas on the images correspond to a strong acoustic return from the bottom. The bright stripes are probably linear outcrops of bare rock, separated by darker stripes which probably represent

sediment-filled grooves between the rock outcrops. The other bright parts of the images are more complex areas of rock outcrop facing towards the track of the sonar vehicle. On the fossil high some of these bright areas can be shown to be outcrops rising above the striated surface, perhaps klippe on a fault surface. Dark areas either lie under the vehicle track or are shadows cast by local highs. The vehicle path runs along the centre of each image. Continuous, mirrored white lines are the sea-surface acoustic reflection.

western RTI about 1 million years ago, and here termed a fossil inside-corner high) and part of another to the north of the transform. These, as well as other inside-corner highs, have been surveyed at lower resolution on previous cruises^{13–15}.

Both active and fossil inside-corner highs at the Atlantis transform are capped by broad, shallow-dipping surfaces which are abnormally smooth compared to typical sea floor near a spreading centre. The surfaces are planar on the fossil highs and curved convex upwards on the active high at the eastern RTI (Fig. 2). Superimposed on these surfaces are corrugations running parallel to the spreading direction with a wavelength of ~ 1 km, a length of up to 8 km, and an amplitude of tens of metres. These can be seen at a lower resolution on the eastern Atlantis RTI in pre-existing bathymetric maps¹⁵. A search through publicly available bathymetric images (RIDGE multibeam bathymetry compilation, available at <http://imager.ldeo.colombia.edu>) found corrugations on a fossil high just south of the Kane transform. No corrugated surfaces can be seen on these existing images of the inside-corner highs at the non-transform offsets that separate the 18 spreading segments between the Kane and Atlantis transforms¹⁵ or in the five segments south of the Kane transform¹⁶. Corrugated surfaces may thus be restricted to the inside-corner highs of transform faults.

Deep-towed side-scan images from the active high and one of the fossil highs show that the corrugated surfaces are striated at a smaller scale parallel to the corrugations, and hence also parallel to the spreading direction (Fig. 3). These striations can be seen to be up to 3 km long, with a wavelength of 50–100 m. The morphology of the striations is similar, on a much larger scale, to slickensides on fault planes. The most likely explanation for the striping seen on the sonar images is that the brighter stripes are exposures of bare rock, and the darker stripes are sediment-filled depressions between them.

The corrugated, striated slip surface at the active inside-corner

high (Fig. 2a) dips away from the spreading axis where it is furthest from the spreading centre. Following the surface towards the spreading axis, there is a smooth transition to horizontal, and then to a dip of 10° – 12° towards the axis. Eventually the surface dips below a rock unit with the morphology of a slipped block having a back-tilted top and convex lower slopes. Side-scan sonar images of the top of this block show that it has a sediment-covered hummocky morphology, characteristic of the median valley floor morphology¹⁰, while images of its convex lower slopes suggest that they are debris flows. By contrast, the corrugated surface on the top of the fossil high (Fig. 2b) is planar and nearly horizontal, with small blocks lying on top of the surface, and small windows through it to rocks beneath.

Our new data show a range of debris flow morphologies within the crustal strip generated at the inside corners. Not only are the inside-corner highs flanked by debris flows, but the area between the active and fossil inside-corner highs also show a wide range of debris flow morphologies. Throughout the whole strip there is no trace of the volcanic morphology characteristic of the median valley floor, except on the top surface of the slipped block. But basalts must outcrop there as they were dredged from several sites in the strip (Fig. 1).

One successful dredge haul was made from each of the striated surfaces imaged by deep-towed side-scan sonar and both contained fragments of serpentinized peridotite. Other dredge hauls of serpentinite were made from near the summit of the active inside-corner high, and in other places in the inside-corner areas (Fig. 1 shows new data as well as earlier hauls^{17,18}). Gabbroic rocks characteristic of the lower crust were represented by one major dredge haul and by samples in other hauls.

The corrugated, striated slip surfaces that we have imaged appear to be analogous to detachment surfaces seen in the extensional tectonic regions of the southwestern United States¹⁹, though much

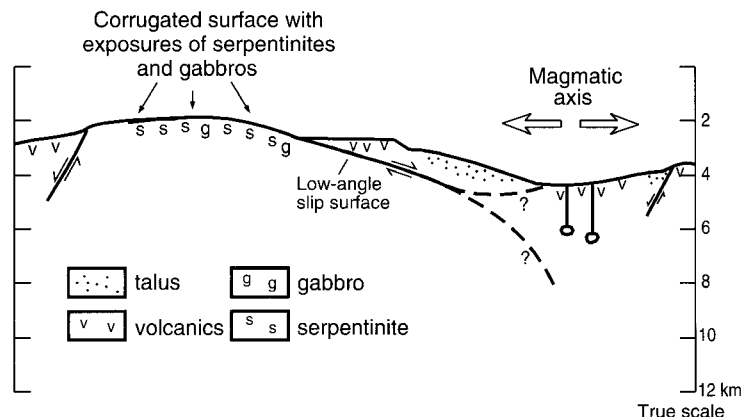


Figure 4 Cartoon cross-section of the active inside-corner high at the eastern Atlantis RTI based on a true-scale topographic profile, showing observations that constrain possible models. The direction taken by the corrugated slip surface is critical (question marks on the figure). The model of Tucholke and Lin³ (lower question mark) would consider the slip surface as a tectonic detachment fault that steepens as it descends through the crust into the upper mantle, detaching much of the lower crust and mantle from the upper crust during spreading. The surface flattens as it spreads away because of isostatic compensation effects. The low angle of the detachment as it emerges from beneath the slipped block must be that of an active part of the fault, because the slipped block is scarcely, if at all, back tilted. This suggests that the fault must at this place lubricated by a very weak material such as serpentinite. Note that though the model of Tucholke and

Lin strictly applies to non-transform offsets, where we have so far failed to find evidence of low-angle slip surfaces, it is based on earlier models that were developed for RTIs⁶⁻⁸. An alternative model (upper question mark) assumes that the slip surface is a major failure plane in a serpentinite-lubricated landslide²¹ and that the failure plane emerges to outcrop on the median valley floor. In this model the inside-corner high would be underlain by thinned, but otherwise nearly complete, ocean crust. Steep tectonic faults penetrate to the mantle, and allow water to percolate downwards to hydrate the mantle into low-density serpentinite. This rises buoyantly up the faults to emerge at the sea floor, where, because of its weakness, it fails at a very low angle, transporting slices of upper crust towards the median valley floor.

less eroded. Analogies include the low dip of the surfaces, the slipped, tilted block on top of the active surface and the presence of plutonic crustal rocks at and below the surfaces. The detachments in the Chemehuevi Mountains in the southwestern United States are also corrugated, with a very similar wavelength and amplitude to those seen here, and with the corrugation axis running parallel to the transport direction²⁰. The 10°–12° maximum dip of the observed sea-floor slip surfaces is shallower than has been previously suggested for inside-corner detachment faults³, but is similar to dips observed on detachment surfaces in the southwestern United States^{19,20}. Slip along such low angle normal faults may be facilitated in oceanic crust by the occurrence of lubricating serpentinite which was sampled on two striated surfaces. The change in dip of the corrugated surface at the active inside-corner high with distance from the spreading axis, and the very low dip on the fossil highs, suggests that rotation of the slip plane occurs with time as extension, uplift and spreading take place.

A number of models may be compatible with our observations. Figure 4 shows a cartoon of our observations on the active high, with some extrapolations. Critical in any interpretation is the behaviour of the corrugated surface as it goes beneath the slipped block. The model of Tucholke and Lin³, based on previous models⁶⁻⁸, would consider the slip surface to be a major detachment between the upper crust and the lower crust/upper mantle, with a radical effect on crustal structure. In its simplest form, this model does not apply here, as the slipped block is made of upper-crustal volcanics, and the model envisages that all of the upper crust is displaced to the outside corners. Previous estimates of the dip of such detachment faults, based on more indirect evidence, suggests dips of 20°–40°. Dips as low as those seen here would require a very low-friction fault rock, such as serpentinite. Another possibility is that the corrugated surface, instead of terminating in the mantle, emerges at the floor of the median valley as a major failure surface in a serpentinite-lubricated landslide zone²¹. This model, however, does not account for the thinned crust deduced for inside-corner highs^{3,6-8}.

Why might such phenomena be confined to inside-corner

regions of RTIs and apparently absent from non-transform offsets and within spreading segments? At slow spreading rates, newly accreted plate cools and thickens rapidly within 15–20 km of the ridge axis. At an RTI, the motion of the juxtaposed thick plate can drag subaxial asthenosphere away from the inside corner. A combination of this induced lateral flow and along-axis flow that compensates for reduced upwelling near a large-offset transform^{22,23} results in greater viscous stresses at a RTI. In comparison, at non-transform offsets the differences in lithospheric thickness across adjacent segment ends are smaller, so that variations in the stresses on the plate are small²⁴. □

Received 12 June; accepted 25 November 1996.

- Karson, J. A. & Dick, H. J. B. *Mar. Geophys. Res.* **6**, 51–98 (1983).
- Severinghaus, J. P. & MacDonald, K. C. *Mar. Geophys. Res.* **9**, 353–367 (1988).
- Tucholke, B. E. & Lin, J. J. *Geophys. Res.* **99**, 11937–11958 (1994).
- Dick, H. J. B. in *Magnetism in the Ocean Basins* (eds Saunders, A. D. & Norry, M. J.) 71–105 (Spec. Publ. 42, Geol. Soc., 1988).
- Cannat, M. H. J. *Geophys. Res.* **98**, 4163–4172 (1993).
- Dick, H. J. B., Bryan, W. B. & Thompson, G. *EOS* **62**, 406 (1981).
- Brown, J. R. & Karson, J. A. *Mar. Geophys. Res.* **10**, 109–138 (1988).
- Mutter, J. C. & Karson, J. A. *Science* **257**, 627–634 (1992).
- Ballard, R. D. & van Andel, T. J. *Geol. Soc. Am. Bull.* **88**, 507–530 (1977).
- Smith, D. K. *et al.* *J. Volcanol. Geotherm. Res.* **67**, 233–262 (1995).
- Macdonald, K. C. & Luyendyk, B. P. *Mar. Geophys. Res.* **7**, 515–535 (1985).
- Pockalny, R. A., Detrick, R. S. & Fox, P. J. *J. Geophys. Res.* **93**, 3179–3193 (1988).
- Pariso, J. E., Sempere, J.-C., Rommevaux, C. *J. Geophys. Res.* **100**, 17781–17794 (1995).
- Cannat, M. *et al.* *Geology* **23**, 49–52 (1995).
- Purdy, G. M., Sempere, J.-C., Schouten, H., Dubois, D. & Goldsmith, R. *Mar. Geophys. Res.* **12**, 247–252 (1990).
- Gente, P. *et al.* *Earth Planet. Sci. Lett.* **129**, 55–71 (1995).
- Miyashiro, A., Shido, F. & Ewing, M. *Contr. Mineral. Petrol.* **23**, 117–127 (1969).
- Shand, S. J. *J. Geol.* **57**, 89–92 (1949).
- Davis, G. A. & Lister, G. S. *Geol. Soc. Am. Spec. Pap.* **218**, 133–159 (1988).
- John, B. E. 313–335 (Spec. Publ. 28, Geol. Soc. London, 1987). (Title?)
- Cann, J. R. *et al.* *EOS* **62**, 406 (1992).
- Parmentier, A. M. & Forsyth, D. W. *J. Geophys. Res.* **90**, 678–684 (1985).
- Blackman, D. K. & Forsyth, D. W. 311–126 (Monogr. 71, Am. Geophys. Union, 1992).
- Blackman, D. K. *Geophys. Res. Lett.* (in the press).

Acknowledgements: We thank J. Goff for advice and criticism, and the staff of RRS Charles Darwin and technicians and engineers from the Southampton Oceanography Centre for assistance. Collection of these data was supported by the UK Natural Environment Research Council BRIDGE program.

Correspondence and requests for materials should be addressed to J.R.C. (e-mail: j.cann@earth.leeds.ac.uk).

2.5-million-year-old stone tools from Gona, Ethiopia

S. Semaw*, P. Renne†, J. W. K. Harris*, C. S. Feibel*, R. L. Bernor‡, N. Fesseha‡ & K. Mowbray*

* Department of Anthropology, Douglass Campus, Rutgers University, New Brunswick, New Jersey 08903, USA

† Berkeley Geochronology Center, 2455 Ridge Road, Berkeley, California 94709, USA

‡ Laboratory of Paleobiology, Department of Anatomy, College of Medicine, Howard University, Washington DC 20059, USA

The Oldowan Stone tool industry was named for 1.8-million-year-old (Myr) artefacts found near the bottom of Olduvai Gorge, Tanzania. Subsequent archaeological research in the Omo (Ethiopia) and Turkana (Kenya) also yielded stone tools dated to 2.3 Myr. Palaeoanthropological investigations in the Hadar region of the Awash Valley of Ethiopia¹, revealed Oldowan assemblages in the adjacent Gona River drainage². We conducted field work in the Gona study area of Ethiopia between 1992 and 1994 which resulted in additional archaeological discoveries as well as radioisotopic age control and a magnetic polarity stratigraphy of the Gona sequence. These occurrences are now securely dated between 2.6–2.5 Myr. The stone tools are thus the oldest known artefacts from anywhere in the world. The artefacts show surprisingly sophisticated control of stone fracture mechanics, equivalent to much younger Oldowan assemblages of Early Pleistocene age. This indicates an unexpectedly long period of technological stasis in the Oldowan.

In 1976 the first archaeological occurrences of stone tools were identified in a fine-grained context at Gona³, and two additional sites were reported later⁴. Fieldwork in 1992–94 increased the number of reported sites (Fig. 1a) and has provided the impetus for a reassessment of the geological context, age and character of the Gona archaeological concentrations.

Strata associated with the Gona sites comprise three sedimentary intervals within the Kada Hadar Member of the Hadar Formation: an upward-coarsening interval of lacustrine and deltaic strata at the base of the sequence, five upward-fining fluvial cycles, and a predominantly fine-grained sequence of fluvial deposits capping the sequence. These sediments record the infilling of a lake basin, subsequent progradation of a delta plain, and cycling of fluvial channel and floodplain environments across the localities. The archaeological occurrences are found in floodplain environments, close to margins of channels that carried the volcanic cobbles used as raw materials for tool manufacture.

Relationships between Gona and Hadar strata are given in Fig. 1b. In the Gona, a series of marker beds consisting of vitric tephra and bentonites (altered vitric tephra) provide local correlation and tie the sequence to the Kada Hadar Member to the east. The lowest of these is a greenish bentonite in the Gona, the Green Marker. It correlates on lithologic and stratigraphic grounds with BKT-2L₁ in the Kada Hadar. A few metres above this is a discontinuous anorthoclase–phyric tephra, which correlates with BKT-2L. The next two markers, vitric tephra AST-1 and AST-2, have not yet been recognized outside the Gona. Between these tephra is a prominent conglomerate termed the Intermediate Conglomerate⁵. Another prominent conglomerate above AST-2 is the Upper Conglomerate, and a bentonite channelled into this conglomerate (cinerite III⁵) we refer to as AST-2.5. In outcrops immediately above this restricted exposure of AST-2.5 is plagioclase–phyric bentonite AST-2.75. This portion of the sequence is capped by a third vitric tephra, AST-3. None of the ASTs have been correlated into the Kada Hadar sequence. However, we have chronostratigraphic data on AST-

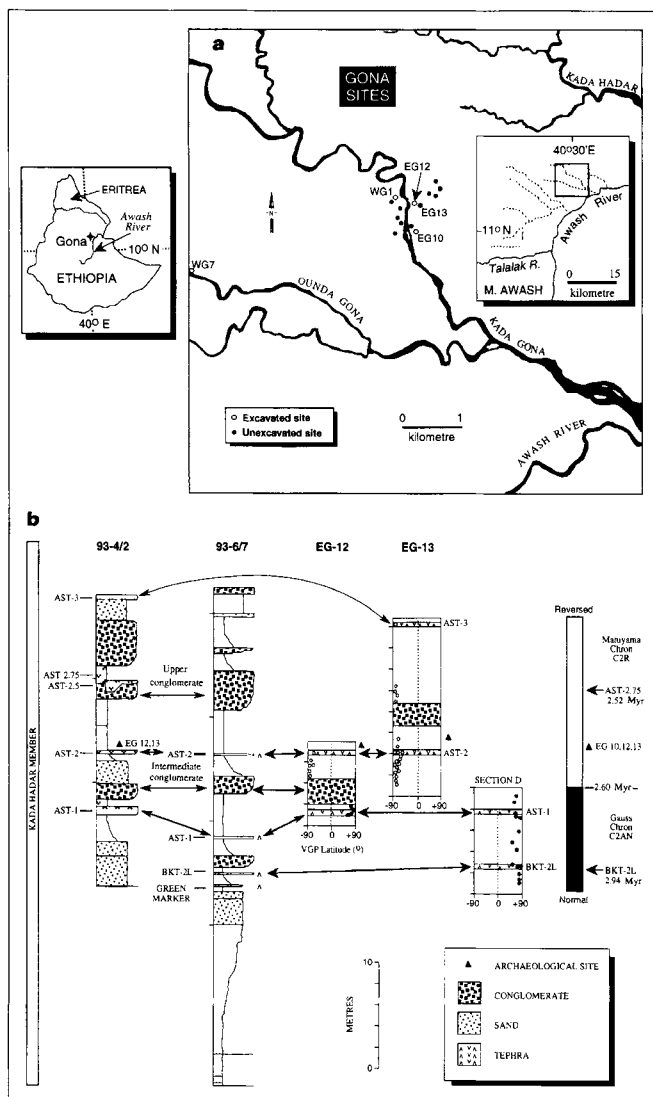


Figure 1 a, Map of the Gona River drainage showing locations of the archaeological sites. b, Stratigraphic context of the Gona sites. Lithostratigraphy and markers from the East Gona exposures; composite sections (93-4/2 and 93-6/7) are correlated with results of magnetic polarity sampling from EG12/EG13 and section D (at 93-6/7). The profiles represent composite sections of the Gona stratigraphic sequence. Significant stratigraphic markers are listed next to the columns and correlations shown with solid lines. Filled circles indicate normal polarity, open circles reversed polarity. Chronostratigraphic context with units of the magnetic polarity timescale (MPTS) and isotopic age determinations are given on the right.

2.75, and magnetic polarity stratigraphy through the sequence to provide additional control.

The age of the Gona artefact sites is constrained by a combination of radioisotopic dating and magnetic polarity stratigraphy (Fig. 1b). Magnetic stratigraphy revealed a transition from normally magnetized basal strata to reversed polarities at the level of the Intermediate Conglomerate, between tephra AST-1 and 2. Tuffs BKT-2L₁ and BKT-L have been identified near the bottom of the Gona sequence on the basis of lithologic similarity and outcrop tracing. In the adjacent Kada Hadar drainage, they underlie a third Bouroukie Tuff, BKT-2u. Ages of 2.95 Myr for BKT-2L and 2.92 Myr for BKT-2u have been recently cited⁶, although no uncertainties were reported, nor have data been published. We report here new data documenting an age of 2.94 Myr for BKT-2L and 2.52 Myr for AST-2.75 (Fig. 2 and Table 1). This establishes the normal magnetozon

Table 1 Ar/Ar analytical data for AST-2.75 and BKT-2L

Lab no. (watts)	⁴⁰ Ar (mol) ($\times 10^{-15}$)	⁴⁰ Ar/ ³⁹ Ar	³⁷ Ar/ ³⁹ Ar	³⁶ Ar/ ³⁹ Ar ($\times 10^{-3}$)	⁴⁰ Ar*/ ³⁹ Ar	% ⁴⁰ Ar*	Age (Myr)	$\pm \sigma$ (Myr)
AST-2.75 (Single crystal total fusion)								
8302-01	1.165	4.856	3.084	5.101	3.604	74.0	2.557	0.311
8302-02	2.014	7.017	2.485	12.385	3.562	50.7	2.527	0.260
8302-03	3.106	6.054	3.030	9.675	3.445	56.8	2.444	0.163
8302-04	1.062	21.970	13.955	63.094	4.489	20.2	3.184	1.710
8302-05	0.651	5.269	2.812	8.941	2.858	54.1	2.028	0.597
8302-06	1.783	7.873	2.472	17.899	2.787	35.3	1.977	0.432
8302-07	0.533	6.698	2.934	12.699	3.187	47.5	2.261	0.857
8302-08	0.504	27.587	0.046	72.863	6.059	22.0	4.297	4.210
8302-09	0.577	4.694	3.120	7.706	2.672	56.8	1.896	0.480
8302-10	0.553	5.456	2.730	6.544	3.748	68.6	2.659	0.503
8302-11	0.341	5.942	3.197	9.146	3.503	58.8	2.485	0.926
8302-12	0.678	6.195	3.497	10.013	3.525	56.7	2.501	0.547
8302-13	2.049	5.610	0.004	6.637	3.649	65.0	2.589	0.181
8302-14	1.188	7.851	2.582	15.958	3.348	42.6	2.376	0.483
8302-15	0.754	19.284	1.172	56.896	2.567	13.3	1.821	1.971
8302-16	0.275	4.465	3.489	1.797	4.224	94.4	2.996	1.176
8302-20	82.311	33.003	0.033	70.249	12.247	37.1	8.674	0.083
8302-21	0.237	15.566	38.207	21.730	12.566	78.4	8.899	1.505
8302-22	0.282	4.903	3.050	4.711	3.763	76.6	2.670	0.474
8302-23	4.541	42.883	1.816	2.477	42.355	98.6	29.823	0.296
8302-24	6.624	35.672	0.816	8.843	33.144	92.9	23.379	0.162
8302-25	0.467	10.666	3.110	23.173	4.077	38.1	2.892	0.483
8302-26	0.704	10.816	4.077	24.397	3.945	36.4	2.799	0.318
8302-27	24.226	40.627	0.010	19.737	34.796	85.6	24.536	0.087
8302-28	0.785	6.732	4.952	12.390	3.480	51.5	2.469	0.198
8302-29	16.982	35.005	0.143	0.447	34.888	99.7	24.601	0.075
8302-30	0.454	5.000	3.201	4.392	3.967	79.2	2.814	0.303
8302-31	6.865	57.853	4.131	176.057	6.178	10.6	4.381	0.454
8302-32	0.258	5.389	3.075	6.967	3.585	66.4	2.543	0.629
Weighted mean								2.517 $\pm$ 0.075
BKL-2L (Single crystal total fusion)								
7201-01	2.099	3.495	0.633	0.344	3.438	98.5	2.936	0.070
7201-02	3.564	3.515	0.230	0.303	3.438	97.9	2.936	0.037
7201-03	2.195	3.541	0.857	0.490	3.458	97.7	2.953	0.057
7201-04	4.744	3.447	0.224	0.072	3.439	99.9	2.936	0.027
7201-05	2.825	3.507	0.231	0.163	3.472	99.1	2.965	0.054
7201-06	4.568	3.469	0.233	0.196	3.424	98.8	2.924	0.033
7201-07	5.078	3.492	0.250	0.216	3.443	98.7	2.940	0.028
7201-08	4.639	3.462	0.228	0.017	3.470	100.4	2.963	0.025
7201-09	2.098	3.699	0.218	0.762	3.485	94.4	2.976	0.059
7201-10	3.603	3.481	0.224	0.125	3.457	99.4	2.952	0.035
7201-14	5.612	3.654	0.227	0.579	3.496	95.8	2.985	0.021
7201-15	5.201	3.552	0.233	0.385	3.451	97.3	2.947	0.020
7201-16	3.695	3.494	0.229	0.377	3.395	97.3	2.899	0.025
7201-17	2.412	3.511	0.227	0.336	3.424	97.7	2.924	0.039
7201-18	6.018	3.488	0.246	0.154	3.457	99.2	2.952	0.017
7201-19	4.206	3.472	0.230	0.106	3.453	99.6	2.949	0.024
7201-20	5.572	3.488	0.231	0.067	3.481	99.9	2.972	0.017
7201-21	2.002	3.574	0.734	0.510	3.475	97.3	2.968	0.045
7201-22	3.935	3.491	0.224	0.310	3.412	97.9	2.914	0.023
7201-24	0.513	3.800	0.205	1.104	3.484	91.8	2.976	0.175
7201-25	7.476	3.561	0.234	0.604	3.395	95.5	2.900	0.013
Weighted mean								2.940 $\pm$ 0.006
BKL-2L (Bulk-step heating)								
(2.1)	0.306	7.275	1.158	19.628	1.559	21.4	1.332	0.619
(2.5)	0.342	4.939	0.860	11.467	1.611	32.6	1.376	0.337
(2.8)	0.700	5.477	0.617	9.346	2.758	50.4	2.356	0.213
(3.0)	0.428	3.810	0.559	0.937	3.572	93.8	3.050	0.364
(3.3)	0.432	3.563	0.526	0.516	3.446	96.8	2.943	0.315
(3.6)	0.441	3.495	0.516	0.902	3.263	93.5	2.787	0.289
(3.9)	0.606	4.047	0.527	2.159	3.445	85.2	2.942	0.173
(4.4)	1.502	3.607	0.396	0.804	3.395	94.2	2.900	0.057
(4.7)	0.361	3.509	0.488	0.485	3.399	97.0	2.902	0.374
(5.1)	0.670	3.627	0.636	1.148	3.332	92.0	2.846	0.127
(5.6)	0.648	3.865	0.597	1.668	3.413	88.4	2.915	0.136
(6.0)	0.665	3.581	0.618	1.085	3.303	92.3	2.821	0.130
(6.5)	0.608	3.432	0.603	0.142	3.432	100.1	2.931	0.219
(7.0)	0.782	3.467	0.604	0.640	3.320	95.9	2.835	0.150
(7.5)	2.301	4.457	0.866	3.643	3.443	77.3	2.941	0.053
(8.0)	3.219	3.422	0.818	0.263	3.403	99.5	2.906	0.026
(8.5)	0.124	2.951	0.342	-1.199	3.327	112.9	2.841	0.804
(8.5)	0.201	3.491	0.359	0.383	3.401	97.5	2.904	0.565
(8.5)	5.930	3.461	0.458	0.152	3.447	99.7	2.943	0.010
Plateau age								2.936 $\pm$ 0.010

at the base of the Gona sequence as the upper Gauss Chron, and the overlying reversed magnetozone as the lowermost Matuyama Chron. The Gauss–Matuyama Chron transition occurred at 2.6 Myr (ref. 7). The two excavated sites EG10 and EG12 directly overlie AST-2 and fall stratigraphically below AST-2.75. They are thus tightly bracketed between 2.6 and 2.5 Myr.

Figure 1a maps the Gona archaeological sites. Systematic excavations at EG10 (13 m²) and at EG12 (9 m²) yielded the largest and most informative artefact assemblages ($n = 2,970$ stone artefacts, 1,114 of these being *in situ*). The artefacts were restricted to a 10-cm interval of a 6-m-thick, well consolidated clay-rich palaeosol. An additional 43 surface and excavated artefacts were recovered in 1994 from WG7, increasing the geographical distribution of the archaeological occurrences to include the Ounda Gona catchment. The fine-grained context, the limited vertical dispersion, the fresh quality of the artefacts and the lack of artefact-size sorting suggests a low-energy depositional environment.

The excavated and surface artefact assemblages at EG10 and EG12 primarily comprise simple cores, whole flakes and flaking debris. Unifacially and bifacially flaked cores comprise the ‘flaked pieces’ category. There are numerous examples of several generations of flake scars on the cores, indicating that Late Pliocene hominids had mastered the skills of basic stone knapping (Fig. 3). Moreover, the large number of well struck flakes with conspicuous bulbs of percussion indicate a clear understanding of conchoidal fracture mechanics. ‘Detached pieces’ are numerically dominant, with values in the range of 75–95%. There are a few ‘pounded pieces’, namely pieces modified or shaped by pounding or battering, like hammerstones, anvils or battered cobbles.

Trachyte was the main raw material source, comprising >70% of the artefacts. Other volcanics such as rhyolite and basalt were also used. The likely sources of raw materials were nearby stream conglomerates. Clasts from the level of the Intermediate Conglomerate show that trachyte cobbles were the most abundant, comprising about 50% of the total sample that also includes chalcedony, breccia and other lavas. The high proportion of trachyte artefacts could be taken to mean an appreciation of the flaking properties and selectivity for this particular raw material over others. There is a good correspondence between the sizes of the stone clasts and the sizes of the excavated stone artefacts. The close proximity of raw material sources may account for the less exhausted nature of the ‘flaked pieces’ and the very low numbers of ‘unmodified pieces’ (manuports) found at the archaeological sites.

The artefact collections from Olduvai Gorge, Koobi Fora and the Omo^{8–11} are the largest and best-studied samples of Oldowan artefacts. The oldest reliably dated among these are those from Lokalalei¹², which are constrained to be younger than the Kalocho Tuff (=Tuff F of the Shungura Fm) at 2.36 ± 0.05 Myr. The Gona artefacts are thus at least 160 Kyr $\pm$ 90 Kyr older than these, the ages being distinguishable with greater than 92% confidence. All these stone assemblages are characterized by simple flaked cores made on cobbles and blocks, and associated flaking debris. The composition of the Gona assemblages is very similar to Plio–Pleistocene sites elsewhere, except for lower diversity of the cores from Gona and the high incidence of utilized pieces and manuports at Olduvai Gorge.

In addition to the lower diversity of the types seen in the Gona core forms, other contrasts exist between these and the Early Pleistocene assemblages. There are no retouched flakes such as light duty flake scrapers at Gona, which are present in small quantities in the Olduvai assemblages. In contrast to these minimal differences, we see strong parallels among all Plio–Pleistocene assemblages dated between 2.5–1.5 Myr. Assemblages from Olduvai and Koobi Fora show a greater degree of core reduction, but knowledge of fundamental conchoidal fracture mechanics suggests that the Gona artefacts are not significantly different. Indeed, the greater core reduction witnessed at Koobi Fora and Olduvai may be due to the considerable distances of these sites from the sources of

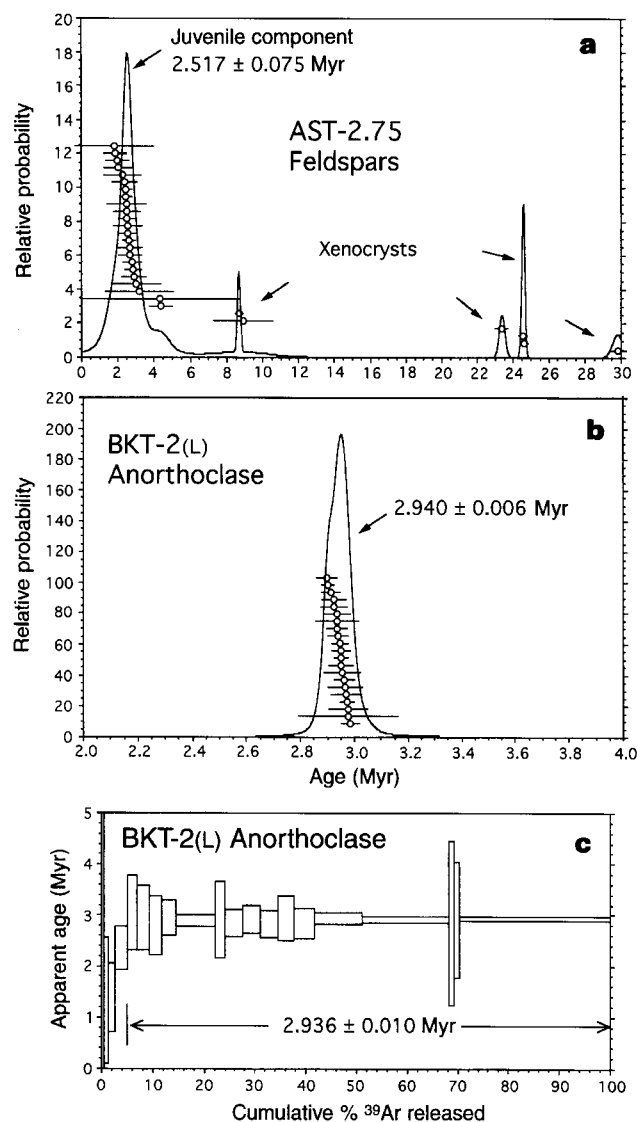


Figure 2 Age–probability plots for **a**, AST-2.75, and **b**, BKT-2L for single-crystal laser fusion $^{40}\text{Ar}/^{39}\text{Ar}$ analyses. **c**, Apparent age spectrum for bulk argon-ion laser heating of BKT-2L.

raw materials compared to Gona.

The presence of large concentrations of stone artefacts at the early Gona sites shows that by 2.5 Myr some populations of Late Pliocene hominids had already mastered the basics of stone tool manufacture. The working edges of the majority of Gona artefacts are very fresh and sharp. Many of the cores show evidence of pitting and bruising. This suggests that in addition to being sources of sharp-edged flakes¹³, the cores were used as multipurpose tools, for example as hammerstones and for other pounding activities.

The Gona evidence extends the known age of the Oldowan Industrial Complex to 2.5 Myr, and obviates the need to posit a ‘pre-Oldowan’¹⁴ or an early facies of the Omo industrial complex^{10,12}. Clearly, all the Oldowan assemblages group together because of the simplicity of craft practices in fashioning simple cores and the resultant flakes. These contrast with Acheulean assemblages that show elements of much more specific and preconceived designs¹⁵. The very early age of the Gona artefacts shows a technological stasis in the Oldowan industrial complex for over a period of 1 million years. The Acheulean appears abruptly at about 1.6–1.5 Myr with large bifacial tool forms such as handaxes and cleavers that were unknown in the Oldowan (*sensu stricto*)^{16–18}.

The hominids responsible for the manufacture of the Gona

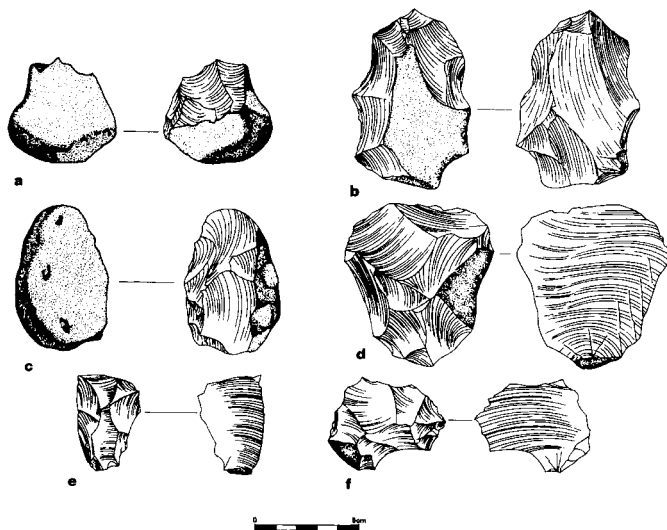


Figure 3 Sketches of a sample of the excavated Gona artefacts. Flaked pieces: **a**, unifacial side chopper, EG12; **b**, discoid, EG10; **c**, unifacial side chopper, EG10. Detached pieces: **d–f**, whole flakes, EG10. Note that the maximum dimension of **d** is as large as some of the flaked pieces.

artefacts remain unidentified. There is, as yet, no evidence of stone artefacts in sediments older than 2.6 Myr within the Gona. Two contemporaneous hominid species *Homo* and *A. aethiopicus* are known elsewhere in eastern Africa from deposits that are comparable in age with the Gona^{19,20}. However, no hominid specimens have yet been found associated with Late Pliocene stone tools.

The sophisticated understanding of conchoidal fracture evidence at Gona implies that the hominids that lived about 2.5 Myr ago were not novices to lithic technology. We predict that even older artefacts will be found. Future research in the Gona study area will be directed towards searching for archaeological evidence in the older deposits and towards assessing contrasting hypotheses linking global climatic changes, the origin of the genus *Homo*, and the beginning of stone-tool manufacture and use^{21,22}. □

Methods

Palaeomagnetic sampling and analysis were done as described previously²³. Ar/Ar dating used methods and facilities described in refs 24, 25. Samples were irradiated in the Oregon State University Triga reactor for 1.5 h using the cadmium-lined CLICKIT facility. Data for BKT-2L and AST-2.75 are presented in Table 1: isotope ratios are corrected for background, mass discrimination (1.00657 ± 0.00151 to 1.00873 ± 0.0010 per atomic mass unit, measured by routine automated analysis of air pipette samples) and radioactive decay. Sensitivities of the mass spectrometer–electron multiplier systems were $(5–10) \times 10^{13}$ nA per mol for argon isotopes. Nucleogenic interference corrections were made based on $(^{39}\text{Ar}/^{39}\text{Ar})_{\text{Ca}} = 0.00764 \pm 0.0000557$, $(^{36}\text{Ar}/^{37}\text{Ar})_{\text{Ca}} = 0.0002705 \pm 0.0000105$, $(^{40}\text{Ar}/^{39}\text{Ar})_{\text{Ca}} = 0.00014 \pm 0.00060$. Calculated ages are based on *J* values (0.0003935 ± 0.0000003 for AST-2.75, 0.0004737 ± 0.0000003 for BKT-2L) derived from replicate analysis of the Fish Canyon sanidine standard, with an age of 27.84 Myr^{24,25}. Age calculations are based on decay constants $\lambda = 5.543 \times 10^{-10} \text{ Y}^{-1}$. Uncertainties in ages reflect uncertainties in isotope abundances and all corrections, but do not reflect uncertainty in the age of the standard. The arithmetic mean and standard error of the mean are 2.58 ± 0.11 Myr, whereas the inverse variance weighted mean is 2.52 ± 0.08 Myr; we adopt the latter as more appropriate because of the heterogeneous precision of individual analyses. Samples with ages shown in italics (AST-2.75) are contaminants and were excluded from the mean age calculations.

Received 18 June; accepted 25 November 1996.

- Johanson, D. C. *et al.* *Am. J. Phys. Anth.* **57**, 373–402 (1982).
- Corvinus, G. & Roche, H. *L'Anthropologie* **80**, 315–324 (1976).
- Harris, J. W. K. *Afr. Arch. Rev.* **1**, 3–31 (1983).

- Harris, J. W. K. & Semaw, S. *Nyame Akuma* **31**, 19–21 (1989).
- Roche, H. & Tierscelin, J. I. C. *heb. Séanc. Acad. Sci. Paris* **284D**, 1871–1874 (1977).
- Kimbel, W. H. *et al.* *Nature* **368**, 449–451 (1994).
- McDougall, I. *et al.* *Geophys. Res. Lett.* **19**, 2349–2352 (1992).
- Leakey, M. D. *Olduvai Gorge Vol. 3* (Cambridge University Press, UK, 1971).
- Isaac, G. L. in *Earliest Man and Environments in the Lake Rudolf Basin* (eds Coppens, Y., Howell, F. C., Isaac, G. L. & Leakey, R. E. F.) 552–564 (University of Chicago Press, Illinois, 1976).
- Chavaillon, J. in *Earliest Man and Environments in the Lake Rudolf Basin* (eds Coppens, Y., Howell, F. C., Isaac, G. L. & Leakey, R. E. F.) 565–573 (University of Chicago Press, Illinois, 1976).
- Merrick, H. V. & Merrick, J. P. S. in *Earliest Man and Environments in the Lake Rudolf Basin* (eds Coppens, Y., Howell, F. C., Isaac, G. L. & Leakey, R. E. F.) 574–589 (University of Chicago Press, Illinois, 1976).
- Kibunjia, M. *J. Hum. Evol.* **27**, 159–171 (1994).
- Toth, N. *J. Archaeol. Sci.* **12**, 101–120 (1985).
- Piperno, M. in *Hominidae: Proc. 2nd Intern. Congr. Hum. Paleont.* 189–195 (Jaca Books, Milan, Italy, 1989).
- Gowlett, J. A. J. in *Stone Age Prehistory* (eds Bailey, G. N. & Callow, P.) 243–260 (Cambridge University Press, UK, 1986).
- Isaac, G. L. & Curtis, G. H. *Nature* **249**, 624–627 (1974).
- Asfaw, B. *et al.* *Nature* **360**, 732–735 (1992).
- Dominguez-Rodrigo, M. *Complutum* **7**, 7–15 (1996).
- Walker, A. *et al.* *Nature* **322**, 517–522 (1986).
- Hill, A. *et al.* *Nature* **355**, 719–722 (1992).
- Vrba, E. S. in *Evolutionary History of the Robust Australopithecines* (ed. Grine, F.) 405–426 (de Gruyter, New York, 1988).
- deMenocal, P. B. *Science* **270**, 53–59 (1995).
- Renne, P. R. *et al.* *Geophys. Res. Lett.* **20**, 1067–1070 (1993).
- Deino, A. & Potts, R. *J. Geophys. Res.* **95**, 8453–8470 (1990).
- WoldeGabriel, G. *et al.* *Nature* **371**, 330–333 (1994).

Acknowledgements: Permission for this research was granted by the CRCCH and the National Museum of Ethiopia in the Ministry of Culture and Information. On behalf of members of the Gona research project, the two principal investigators (S.S. and J.W.K.H.) thank the Government of Ethiopia and the Afar People. We also thank J. D. Clark, T. White, and F. C. Howell and S. Asrat for their help; B. Asfaw, Y. Beyene and G. WoldeGabriel for encouragement, advice and laboratory assistance at the Museum in Addis; the NTO of Ethiopia and drivers; Y. Haile Selassie, A. Ademassu, J. Haile-Mariam and J. Wynn for overall support; M. Kahasa, A. Asfaw, A. Humet, A. Habib and the Afars at Eloha, Busidima and Talalak for support in the field; Y. Kanaa, M. Siegel and P. Jung for illustrations; T. Assebework and S. Eshete for fieldwork; J. Butterworth for palaeomagnetic analyses; and T. White, R. Blumenshine, D. Lieberman, M. Dominguez-Rodrigo and M. Rogers for comments on the manuscript. We thank the NSF, the L. S. B. Leakey Foundation, the Boise Fund, Ann and Gordon Getty Foundation, D. Holt, Z. Zelazo, and Rutgers University for financial support.

Correspondence and requests for materials should be addressed to S.S. (e-mail: semaw@eden.rutgers.edu).

Circumsporozoite protein is required for development of malaria sporozoites in mosquitoes

Robert Ménard[†], Ali A. Sultan^{*}, Claudio Cortes[†], Rita Altszuler[†], Melissa R. van Dijk[‡], Chris J. Janse[‡], Andrew P. Waters[‡], Ruth S. Nussenzweig[†] & Victor Nussenzweig^{*}

^{*} Michael Heidelberger Division of Immunology, Department of Pathology, Kaplan Cancer Center, New York University Medical Center, New York, New York 10016, USA

[†] Department of Medical and Molecular Parasitology, New York University Medical Center, New York, New York 10016, USA

[‡] Department of Parasitology, University of Leiden, 2300 RC Leiden, The Netherlands

Malaria parasites undergo a sporogonic cycle in the mosquito vector. Sporozoites, the form of the parasite injected into the host during a bloodmeal, develop inside oocysts in the insect midgut, then migrate to and eventually invade the salivary glands. The circumsporozoite protein (CS), one of the major proteins synthesized by salivary gland sporozoites¹, is a surface-associated molecule which is important in sporozoite infectivity to the host². Here, by gene targeting, we created *Plasmodium berghei* lines in which the single-copy CS gene was disrupted. The CS(–) and wild-type parasites produced similar numbers of oocysts of comparable size in the mosquito midgut. In the CS(–) oocysts, however, sporozoite formation was profoundly inhibited. CS therefore appears to have a pleiotropic role and to be vital for

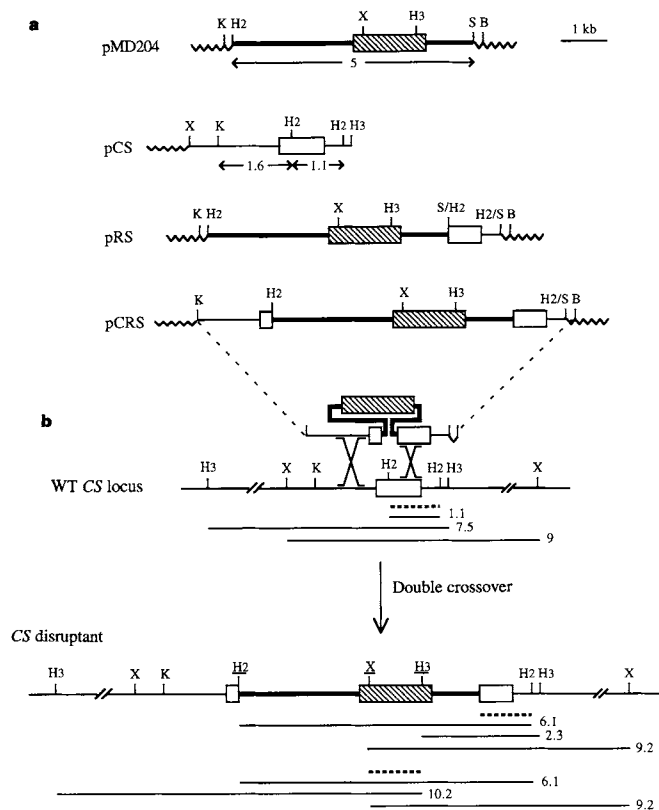


Figure 1 Gene targeting at the CS locus of *Plasmodium berghei*. **a**, Plasmid constructs and replacement plasmid pCRS. Genes are symbolized by boxes. Thick lines, 5' and 3' untranslated regions (UTR) of *DHFR-TS*; thin lines, 5' and 3' UTR of CS; wavy lines, plasmid vector sequences. The sizes of the restriction fragments are indicated in kilobases (kb). Abbreviations: B, *Bam*HI; H2, *Hinc*II; H3, *Hind*III; K, *Kpn*I; S, *Sma*I; X, *Xba*I. **b**, Wild-type (WT) CS locus and CS locus generated by a double crossover occurring between the CS sequences flanking the cassette in the *Kpn*I-*Bam*HI fragment of plasmid pCRS and their homologues in the wild-type genome (CS disruptant). The underlined restriction sites in the CS disruptant are brought by the transformed DNA and the others by the endogenous CS locus. The CS and the *DHFR-TS* probes used in genomic Southern hybridization are symbolized by dashed bold lines. Restriction fragments of the wild type and of the expected recombinant lines detected by the probes are shown below the corresponding locus. Their size is indicated in kb.

malaria parasites in both the vector and the host: in mosquitoes, CS is essential for sporozoite development within oocysts, and in the vertebrate host it promotes sporozoite attachment to hepatocytes³⁻⁷.

Systems for stable transformation of malaria parasites and targeted integration of exogenous DNA into their genome have been developed⁸⁻¹² and have paved the way for a genetic approach to *Plasmodium* biology and pathogenicity. *P. berghei*, a rodent malaria parasite, constitutes a good model for such studies inasmuch as the entire parasite life cycle is easily maintained *in vivo*, and stable transformants can be readily selected and cloned^{10,12}. CS is a single-copy gene¹³ and malaria parasites are haploid during their entire life cycle in the vertebrate host¹⁴, hence a single targeting event might generate CS(−) parasite lines. We therefore constructed the replacement plasmid pCRS in which the CS coding region was interrupted by a selectable cassette containing a pyrimethamine-resistant dihydrofolate reductase thymidylate synthase (*DHFR-TS*) mutant gene constitutively expressed by its flanking regions¹⁰ (Fig. 1a). A double crossover occurring between the homologous CS sequences in the construct and in the genome was expected to lead to the replacement of the wild-type CS by the disrupted copy (Fig. 1b).

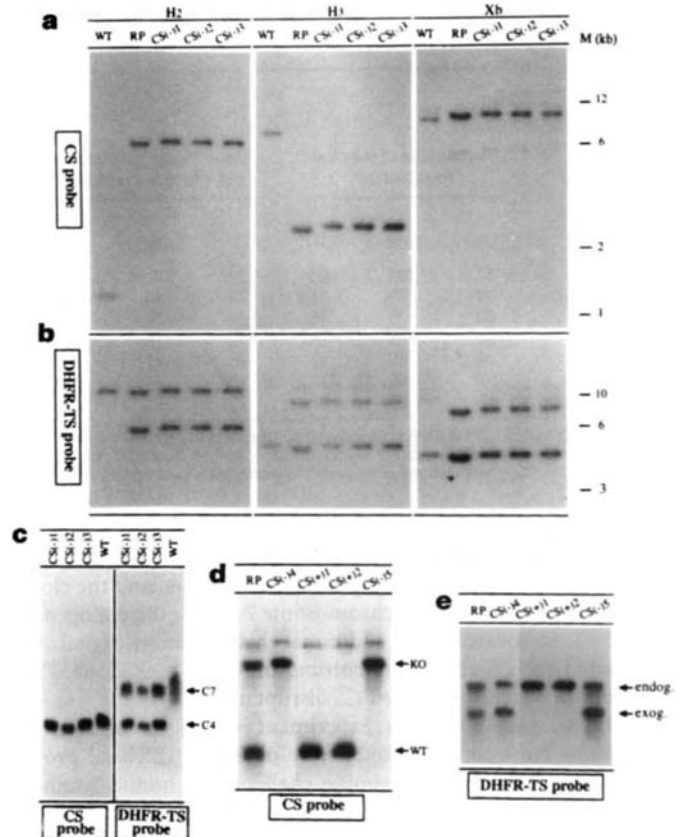


Figure 2 **a** and **b**, Genomic Southern blot hybridization of the wild-type *P. berghei* strain (WT), the resistant parasite population (RP) and of the clones obtained in the first transformation experiment analysed with the CS or the *DHFR-TS* probe, respectively. **c**, Clamped homogenous electric fields gel electrophoresis of the chromosomes of the WT parasites and of the clones from the first experiment probed with the CS or the *DHFR-TS* probes. **d** and **e**, Genomic Southern blot hybridization of the RP and of the clones obtained in the second transformation experiment digested with *Hinc*II and analysed with the CS or the *DHFR-TS* probe, respectively. Molecular size standards are indicated on the right (in kb); C4 and C7, chromosomes 4 and 7; KO, CS gene knockout; endog., endogenous *DHFR-TS* gene; exog., exogenous *DHFR-TS* copy originating from the transformed DNA.

In a first transformation experiment, we used the target DNA as a linear fragment having the CS sequences at its ends to favour integration into the CS genomic locus. Plasmid pCRS digested with the restriction enzymes *Kpn*I and *Bam*HI was introduced by electroporation into *P. berghei* merozoites and transformed parasites were selected in rats with pyrimethamine. Resistant parasites were cloned by limiting dilution into 10 rats, and three parasite clones were obtained at day 36 post-electroporation. The CS locus of parasites of the resistant population and of the clones was analysed by genomic Southern hybridization. When using a CS probe (Fig. 2a), the resistant population and each of the clones appeared as homogeneous populations displaying the pattern indicative of the replacement of the wild-type CS by a single copy of the disrupted CS, with no trace of parasites having a wild-type CS locus. When using a *DHFR-TS* probe (Fig. 2b), a single band corresponding to the endogenous single-copy *DHFR-TS* was detected in the wild type. This band was also found in the resistant population and in each of the clones, named CS(−)1, CS(−)2 and CS(−)3, together with an additional band whose size was predicted by the expected CS allelic exchange. The integration of the linear fragment into the CS locus was confirmed by clamped homogenous

Table 1 Oocyst development and sporozoite formation in CS(+) and CS(−) populations

Parasite population	Oocyst development									Sporozoite development					
	Percentage of infected mosquitoes*			Mean number of oocysts per infected mosquito*			Mean oocyst size*			Percentage of sporozoite-containing midguts*		Percentage of sporozoite-containing salivary glands*		Parasite infectivity to animal†	
	D7	D12	D17	D7	D12	D17	D7	D12	D17	D14	D18	D14	D18	D17	D21
WT	82	100	60	32	41	24	13	24	34	75	78	55	64	2/2	3/3
CS(+)-1	20	70	74	14	45	45	11	31	36	100	74	80	60	2/2	3/3
CS(+)-2	30	76	95	16	39	20	9	28	36	95	95	41	50	2/2	3/3
CS(−)-1	82	75	75	12	12	56	11	17	22	0	0	0	0	0/4	0/2
CS(−)-2	74	85	82	45	53	28	13	27	35	0	0	0	0	0/3	0/2
CS(−)-4	50	65	75	45	64	49	10	25	35	0	0	0	0	0/2	0/3
CS(−)-5	40	95	94	23	31	54	11	29	35	0	0	0	0	0/2	0/3

* Mean value obtained from at least three independent feeding experiments and involving examination of 10 mosquitoes at each time point. Mean oocyst size is expressed in μm .

† Number of infected animals/number of animals exposed to infected mosquitoes. Parasites were first detected in the animal's blood (percentage of infected erythrocytes >0.01) between D2 and 4 with the CS(+) populations and remained undetectable with the CS(−) lines. D, day.

electric fields gel electrophoresis of the chromosomes (Fig. 2c). After hybridization to the *DHFR-TS* probe, the wild type and the clones showed a positive signal on chromosome 7, where the endogenous *DHFR-TS* is located. An additional hybridization signal was detected in the CS-containing chromosome 4 of each clone. Thus the 3 clones were the expected CS disruptants.

A second transformation experiment was done in identical conditions. Four parasite clones were obtained which all proved resistant to a new pyrimethamine challenge. Genomic Southern hybridization using the CS (Fig. 2d) and the *DHFR-TS* (Fig. 2e) probes indicated that the resistant population was a mixture of parasites having either a wild-type or a disrupted CS locus. Two clones, named CS(−)4 and CS(−)5, had the pattern diagnostic of the expected double crossover event. The two other clones, named CS(+)-1 and CS(+)-2, had a wild-type CS locus and only one *DHFR-TS* copy. Thus they arose either from a spontaneous *DHFR-TS* mutation or from a gene replacement of the endogenous *DHFR-TS* by the resistant form borne by plasmid pCRS.

The phenotype of the wild-type population, clones CS(+)-1 and CS(+)-2 used as internal controls, clones CS(−)-1 and CS(−)-2 originating from the first experiment, and clones CS(−)-4 and

CS(−)-5 originating from the second experiment, were all examined. The duration of the schizogonic blood cycle in rats or hamsters and the level of gametocyte production were similar in all parasite populations. To study their sporogonic cycle, *Anopheles stephensi* mosquitoes were fed on infected animals. Similar numbers of morphologically mature ookinetes were detected in all parasite populations by Giemsa staining of mosquito midgut contents 18 h post-feeding (pf). Oocyst development was examined at 7, 12 and 17 days pf, in at least three independent experiments involving more than 10 mosquitoes at each time point (Table 1). The percentage of infected mosquitoes, the number of midgut oocysts per infected mosquito, and the mean oocyst size were similar in the wild-type population, in the CS(+) clones and in each of the CS(−) clones.

As the *P. berghei* oocyst matures, sporozoites are formed and bud off from the central sporoblastoid body of the oocyst "like rays from the sun"¹⁵ (Fig. 3a, c and 4). Before day 10 pf, there was no morphological difference between oocysts of the CS(+) and CS(−) parasite populations by light microscopy. In CS(+) populations, sporozoite-containing oocysts were seen as early as day 11 pf, and at day 14 pf more than 70% of these oocysts were filled with

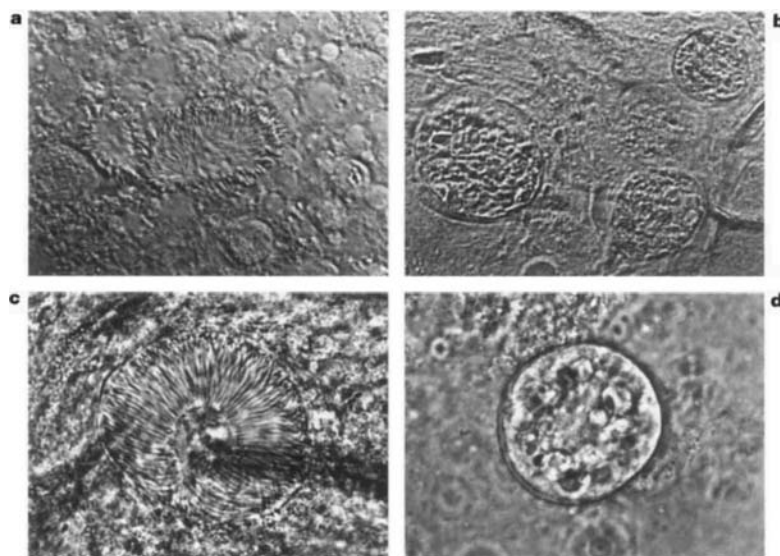


Figure 3 **a** and **b**, Phase-contrast microscopic examination at day 12 pf of mosquito midgut oocysts of the CS(+) wild-type population and of the CS(−)2 clone, respectively. **c** and **d**, Light microscopic examination at day 18 pf of an

oocyst of the CS(+) wild-type population and of the CS(−)2 clone, respectively. No sporozoites can be seen in the highly vacuolated CS(−) oocysts.

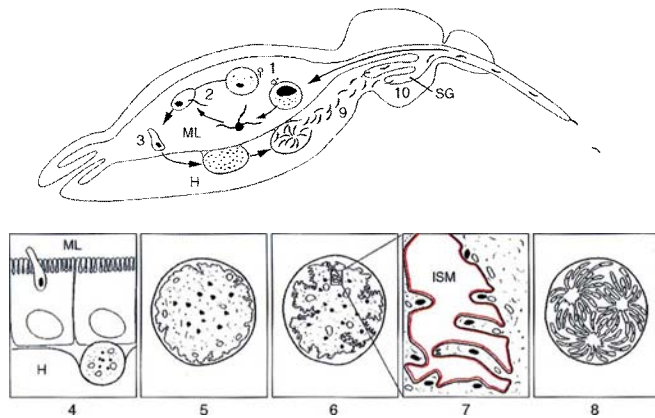


Figure 4 The developmental cycle of *Plasmodium berghei* in *Anopheles stephensi*^{15,26}. (1) Shortly after the mosquito ingests an infected blood meal, male and female gametocytes are liberated from the red blood cells within the midgut lumen and mature into gametes. (2) Fertilization occurs rapidly, generating a zygote (1–5 h). (3) The zygote then elongates to become an ookinete (10–24 h). (4) The motile ookinete penetrates between or within midgut epithelial cells (24–40 h) and moves to the space between epithelial cells and the basal lamina where it transforms into an oocyst (30–40 h) which protrudes into the mosquito haemocoel (body cavity). The growth of the oocyst over the next 6–14 days ends with the release of 5–10,000 motile sporozoites. (5) The young oocyst undergoes nuclear division while its plasma membrane retracts from the capsule and gives rise to invaginations and subcapsular vacuoles. (6) Vacuoles coalesce into large clefts that penetrate and divide the cytoplasm, forming the sporoblast. (7) Sporozoites evaginate along the surface of the CS-bearing membranes (red line) of the sporoblast (beginning on day 10); a nucleus and various cytoplasmic components of the sporoblast are transferred into the sporozoite during the budding process. (8) The mature oocyst filled with needle-shaped sporozoites releases sporozoites from day 11. (9) Sporozoites migrate to and invade salivary glands (beginning on day 12). (10) Sporozoites contained within the acinar cells of the glands are injected into the vertebrate host during subsequent feedings (beginning on day 13). ML, midgut lumen; H, haemocoel; SG, salivary glands; ISM, intersporozoite matrix.

numerous sporozoites (Table 1). In contrast, starting at day 11 pf, CS(–) oocysts displayed a highly vacuolated structure (Fig. 3b, d). Sporozoites could not be seen in any oocyst of these clones up to day 24 pf. However, a few sporozoites could occasionally be detected by light microscopy in the material concentrated from extracts of mosquito midguts containing the CS(–) clones. Their numbers were too low to allow proper quantification.

At days 14 and 18 pf, more than 50% of the salivary glands of mosquitoes harbouring the CS(+) populations contained tens of thousands of sporozoites (Table 1). In contrast, sporozoites were never detected within or in the vicinity of salivary glands of mosquitoes containing either CS(–) clones. To test whether the few sporozoites produced by the CS(–) clones could nonetheless infect a vertebrate host, mosquitoes were fed at days 17 and 21 pf on naive hamsters. Infected red blood cells were found in all control animals exposed to mosquitoes infected with CS(+) populations, whereas no parasite was detected in the blood of any of the animals exposed to mosquitoes containing CS(–) populations.

Many lines of evidence indicate a major role of CS in sporozoite attachment to hepatocytes of the vertebrate host^{3–7}. This function is thought to be mediated by a heparan sulphate proteoglycan-binding domain homologous to the type 1 repeats of thrombospondin (TSP), an extracellular matrix protein that can promote cell attachment and locomotion¹⁶. The identical phenotype of the independently generated CS(–) clones described here provides strong evidence that CS is also crucial for sporozoite development in oocysts. Immunolabelling has shown that CS synthesis starts in

early oocysts before sporozoites are formed^{17–21}. CS of various *Plasmodium* species first appears in the plasma membrane of the unsegmented oocyst, from day 7 after the infective blood meal. Later, CS covers the membranes that delineate invaginations and clefts in the sporoblast cytoplasm and that eventually enclose sporozoites. Surface-associated CS might have an early regulatory function in oocyst growth, reminiscent of the role of secreted and cell-associated TSP in cell division^{22,23}. The TSP type 1 repeats-like domain of CS (the conserved region II+) might also bind to receptors in the intersporozoite matrix of the oocyst and guide the membrane movements that occur during sporogony. In support of this idea, the UNC-5 protein of *Caenorhabditis elegans*, which also contains TSP type 1 domains, is known to act as a transmembrane ligand that guides cell migration²⁴.

Mutagenesis of selected domains of CS should help us to understand the molecular basis underlying the two apparently unrelated CS functions, sporozoite morphogenesis and attachment to hepatocytes, and may provide a rationale for developing anti-CS agents to block the life cycle of malaria parasites in both mosquitoes and humans. □

Methods

Plasmid constructions. Plasmid pMD204 contains the mutated copy of the *DHFR-TS* gene of *P. berghei* that confers resistance to pyrimethamine, as well as 2.5 kb of upstream and 1 kb of downstream sequence¹⁰. CS sequences were obtained from plasmid pCS, a derivative of plasmid p9.5 (ref. 25) which contains the CS coding region and flanking sequences of *P. berghei* strain NK65. Plasmid pRS was constructed by cloning the 1.1-kb *HincII* fragment of pCS containing the 3' part of CS and 400 bp of downstream sequence into *SmaI*-cut pMD204. Plasmid PCRS was obtained by inserting the 1.6-kb *KpnI*–*HincII* fragment of plasmid pCS containing the 5' part of CS and 1.4 kb of upstream sequence into plasmid pRS treated with the same restriction enzymes.

Transformation experiments and selection of the CS(–) and CS(+) clones. Merozoites (10^9) of *P. berghei* strain NK65 were transformed by electroporation¹⁰, with 15 µg of plasmid pCRS digested with *KpnI* and *BamHI* and injected intravenously (i.v.) into rats. Recipient rats received a daily intraperitoneal injection of pyrimethamine (25 mg per kg of body weight) starting 30 h after electroporation for five consecutive days. In both experiments, parasitaemia of these rats was ~1% at 4 h after injection and undetectable (<0.01%) 6 days post-electroporation (pe). A resistant population (RP) emerged at day 8. The rats were again treated for two consecutive days with pyrimethamine. In the first experiment, the RP was passed three consecutive times by i.v. injection into new animals at days 14, 21 and 25 pe. Parasites were then cloned by limiting dilution into 10 rats at day 26 pe, and three parasite clones, CS(–)1, CS(–)2, and CS(–)3, were obtained at day 34 pe. In the second experiment, the RP was cloned at day 10 pe. Four clones, CS(–)4, CS(–)5, CS(+)1 and CS(+)2, were obtained at day 18 pe.

Southern-blot analysis of parasite genomic DNA. Parasite DNA was purified from rat blood free of white blood cells¹⁰, digested with restriction enzymes, size-fractionated in a 0.7% agarose gel, transferred onto nylon membranes and analysed by hybridization to the CS probe (corresponding to the 1.1-kb *HincII* fragment of CS) or the *DHFR-TS* probe (corresponding to the 1.2-kb *XbaI*–*HindIII* internal fragment of *DHFR-TS*). The probes were gel-purified and labelled by random priming with ³²P-labelled deoxyadenine 5'-triphosphate (dATP). Membranes were washed at a final stringency of $0.1 \times \text{SSC}$ –0.1% SDS for 1 h at 65 °C.

Analysis of parasite development in mosquitoes. *Anopheles stephensi* mosquitoes were fed for 15 min on infected rats having a high proportion of differentiated gametocytes and microgametocytes that exflagellated *in vitro*. At days (D) 7, 12, 14, 17 or 18, mosquitoes were dissected and their midguts and salivary glands examined under light microscopy (magnification $\times 40$). Mosquito midguts were also isolated, pooled and triturated in a loose-fitting grinder. The fluid released from the midguts was centrifuged, the supernatant collected and examined by light microscopy for the presence of sporozoites. To assess parasite infectivity to the host, infected mosquitoes at days D17 and D21 post-feeding were allowed to bite naive hamsters for 15 min and the parasitaemia was checked daily for 12 d.

Received 23 October; accepted 29 November 1996.

1. Yoshida, N., Potocnjak, P., Nussenzweig, V. *et al.* *J. Exp. Med.* **154**, 1225–1236 (1981).
2. Nussenzweig, V. & Nussenzweig, R. S. *Cell* **42**, 401–403 (1985).
3. Cerami, C. *et al.* *Cell* **70**, 1021–1033.
4. Pancake, S. J., Holt, G. D., Mellouk, S. & Hoffman, S. L. *J. Cell Biol.* **117**, 1351–1357 (1992).
5. Frevert, U. *et al.* *J. Exp. Med.* **177**, 1287–1298 (1993).
6. Cerami, C., Frevert, U., Sillis, P., Takacs, B. & Nussenzweig, V. *J. Exp. Med.* **179**, 695–701 (1994).
7. Sillis, P. *et al.* *J. Exp. Med.* **180**, 297–306 (1994).
8. Goonewardene, R. *et al.* *Proc. Natl Acad. Sci. USA* **90**, 5234–5236 (1993).
9. Wu, Y., Sifri, C. D., Lei, H.-H., Su, X.-Z. & Wellems, T. E. *Proc. Natl Acad. Sci. USA* **92**, 973–977 (1995).
10. van Dijk, M. R., Waters, A. P. & Janse, C. J. *Science* **268**, 1358–1362 (1995).
11. Wu, Y., Kirkman, L. A. & Wellems, T. E. *Proc. Natl Acad. Sci. USA* **93**, 1130–1134 (1996).
12. van Dijk, M. R., Janse, C. J. & Waters, A. P. *Science* **271**, 662–665 (1996).
13. Osaki, L. S., Svec, P., Nussenzweig, R. S., Nussenzweig, V. & Godson, G. N. *Cell* **34**, 815–822 (1983).
14. Walliker, D. in *Molecular Biology of Parasites* (eds Guardiola, J., Luzzato, L. & Trager, W.) 53–61 (Raven, New York, 1983).
15. Vanderberg, J., Rodin, J. & Yoeli, M. *J. Protozool.* **14**, 82–103 (1967).
16. Prater, C. A., Plotkin, J., Jaye, D. & Frazier, W. A. *J. Cell Biol.* **112**, 1031–1040 (1991).
17. Posthuma, G. *et al.* *Eur. J. Cell Biol.* **46**, 18–24 (1988).
18. Hamilton, A. J., Davies, C. S. & Sinden, R. E. *Parasitology* **96**, 273–280 (1988).
19. Nagasawa, H. *et al.* *Exp. Parasitol.* **66**, 27–34 (1988).
20. Simonetti, A. B., Billingsley, P. F., Winger, L. A. & Sinden, R. E. *J. Euk. Microbiol.* **40**, 569–576 (1993).
21. Boulanger, N., Charoenvit, Y., Kretzli, A. & Betschart, B. *Parasitol. Res.* **81**, 58–65 (1995).
22. Majack, R. A., Coates Cook, S. & Bornstein, P. *J. Cell Biol.* **101**, 1059–1070 (1985).
23. Majack, R. A., Goodman, L. V. & Dixit, V. M. *J. Cell Biol.* **106**, 415–422 (1988).
24. Leung-Hagstjeijn, C. *et al.* *Cell* **71**, 289–299 (1992).
25. Eichinger, D. J., Arnot, D. E., Tam, J. P., Nussenzweig, V. & Enea, V. *Mol. Cell Biol.* **6**, 3965–3972 (1986).
26. Syafruddin, Arakawa, R., Kamimura, K. & Kawamoto, F. *J. Protozool.* **39**, 333–338 (1992).

Acknowledgements: We thank S. Gantt for critically reviewing the manuscript, and S. Sidjanski and J. Ramesar for technical assistance. This work was supported by a 'New Initiative in Malaria Research' grant from the Burroughs Wellcome Fund and a grant from the Institute of Allergy and Infectious Diseases of the NIH. M.R.D., C.J.J. and A.P.W. are supported by grants from the INCO-DC programme of the DG XII of the European Union and from the TDR programme of the WHO. A.A.S. is a recipient of a Fogarty international research fellowship and R.M. of an EMBO fellowship.

Correspondence and requests for materials should be addressed to R.M. (e-mail: (menarr01@mcrc6.med.nyu.edu)).

Cleavage of syntaxin prevents G-protein regulation of presynaptic calcium channels

E. F. Stanley & R. R. Mirotznik

Synaptic Mechanisms Section, National Institute of Neurological Diseases and Stroke, Bld 36, Rm 5A25, National Institutes of Health, Bethesda, Maryland 20892, USA

Neurotransmitter release into the synapse is stimulated by calcium influx through ion channels that are closely associated with the transmitter release sites^{1,2}. This link may involve the membrane protein syntaxin, which is known to be associated with the release sites and to bind to the calcium channels^{3,4}. There is evidence that presynaptic calcium channels are downregulated by second messenger pathways involving G proteins^{5,6}. Here we use the patch-clamp technique to test whether calcium current is regulated by G proteins in a vertebrate presynaptic nerve terminal⁷, and whether this regulation is affected by the linkage to syntaxin. The calcium current in the nerve terminal showed typical G-protein-mediated changes in amplitude and activation kinetics which were reversed by a preceding depolarization. These effects of the G protein were virtually eliminated if syntaxin was first cleaved with botulinum toxin C1. Our findings indicate that this sensitivity of the current to modulation by G proteins requires the association of the presynaptic calcium channel with elements of the transmitter release site, which may ensure that channels tethered at release sites^{2,8} are preferentially regulated by the G-protein second messenger pathway.

Because the calyx-type nerve terminal of the chick ciliary ganglion synapse is large enough to allow the use of patch-clamp techniques⁹, calcium channels in this presynaptic nerve terminal

have been better characterized than in any other synapse: for example, they are high-voltage activated, show little voltage-dependent inactivation, are blocked by ω -conotoxin GVIA (refs 7, 10), are localized on the nerve terminal transmitter release face, and have a unitary conductance of 12 pS (ref. 11), consistent with inclusion within the N-type group^{7,10}.

We tested for calcium-channel regulation by introducing guanosine-5'-O-[3-thiotriphosphate] (GTP- γ S) in the patch solution. This analogue tonically downregulates calcium channels through G-protein-dependent pathways in cell somata^{12–14}. The membrane seal was ruptured and brief depolarizing pulses were given to test the effect of GTP- γ S infusion on inward calcium currents. As the cell was perfused, there was a progressive slowing in the rate of current activation (Fig. 1a) as the channels shifted into a 'reluctant'¹⁵ state, a shift that could be reversed by a strong depolarizing prepulse¹⁴; recovery was maximal after a prepulse duration of more than 15 ms (Fig. 1b). Thus, consistent with findings in neuron cell bodies, presynaptic N-type calcium channels are sensitive to control by the G-protein pathway. This mechanism may underlie the modulation of calcium influx and transmitter release in this synapse by exposure to somatostatin¹⁶.

We next examined the effect of botulinum toxin C1 (BTC1) on presynaptic calcium channels by treating the chicks *in ovo*, 16 to 20 h before recording. The toxin eliminated all movement of the chicks within about 8 h. BTC1 had no obvious effect on the voltage dependence of the presynaptic calcium current (Fig. 2a, left), consistent with findings on currents recorded in the neuron soma¹⁷, nor did it markedly affect the steady-state inactivation curve (Fig. 2a, right).

We then tested BTC1-treated terminals for G-protein modulation. In contrast to the response in controls (Fig. 1a), GTP- γ S did not cause a slow increase in the current activation time constant (Fig. 2b, right), indicating that the GTP analogue could not shift the channels into the reluctant state. We investigated this further using a depolarizing prepulse (Fig. 2c). In the absence of GTP- γ S, the prepulse had no effect on the current in either the control or BTC1-treated nerve terminals. With GTP- γ S, the prepulse recruited about 35% of the maximum calcium current in control terminals (Fig. 1b) but very little in the BTC1-treated terminals, as shown by comparison of either current amplitudes or activation time constants (Fig. 2c, right, and legend). Thus, BTC1 virtually eliminated the sensitivity of calyx calcium channels to activated G proteins.

The simplest interpretation of our results is that BTC1 cleaves a protein that is necessary for G-protein-mediated modulation of presynaptic calcium channels. The botulinum toxins are highly specific and only target three known neuronal proteins. All of these express a SNARE-recognition motif¹⁸ and are critical for transmitter release. BTC1 is unusual in that it cleaves two of these proteins, syntaxin and SNAP-25, in mammalian cells^{19,20}, both of which are believed to interact with N-type calcium channels²¹. Syntaxin is severed close to its membrane-attachment point^{22,23} removing the large N-terminal segment containing most of the molecular binding sites^{24,25}. In contrast, only a very short segment of the C-terminal region of SNAP-25 is cleaved^{19,20}.

We used computer-enhanced immunofluorescence microscopy to examine the effect of the toxin on release-site-associated proteins in the calyx nerve terminal. Control calyces exhibited prominent anti-syntaxin staining on the surface membrane, with clustered staining at the transmitter release face but very little staining in the cytoplasm (Fig. 3A, a). After BTC1 treatment, surface and clustered staining decreased markedly, whereas cytoplasmic staining increased (Fig. 3A, b and legend). These results are consistent with cleavage of the exposed cytoplasmic region of syntaxin, to which our antibody must bind, from the membrane-immersed stub²². We tested for SNAP-25 cleavage with a polyclonal antibody against the vulnerable C-terminal region of the protein and a monoclonal antibody against the resistant N-terminal region (Fig.

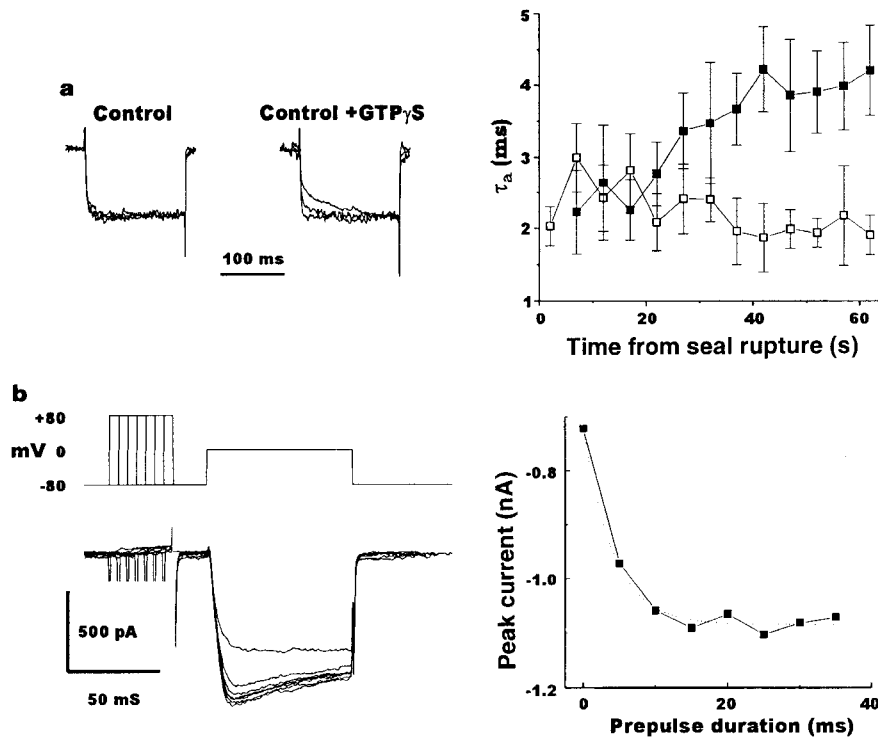


Figure 1 Effect of GTP- γ S on the presynaptic calcium current. **a**, Wash-in effect on the activation time constant (τ_a). Left, superimposed traces at 10 (largest current), 35 and 65 s after membrane-seal rupture in a control and a (control + GTP- γ S)-treated calyx. Current traces have been normalized for comparison of activation rates. Right, change in τ_a in untreated ($\square$) and GTP- γ S-treated ($\blacksquare$) calyces. Each line is the mean $\pm$ s.e. for four calyces. The increase in τ_a with GTP- γ S was complete after ~ 1 min. Holding and step potentials were -80 and $+20$ mV

respectively. **b**, Recruitment of reluctant channels by a depolarizing prepulse. Left, voltage protocol and, below, superimposed current traces in a calyx nerve terminal. A test pulse was preceded by a depolarizing pulse of varying duration to $+80$ mV, beyond I_{Ca} reversal. The shortest prepulse corresponds to the evoked current with the smallest amplitude. Right, peak I_{Ca} plotted against prepulse duration. Data were fitted with a single exponential, with a time constant of 4.1 ms. Maximum recruitment was 35% of the total current.

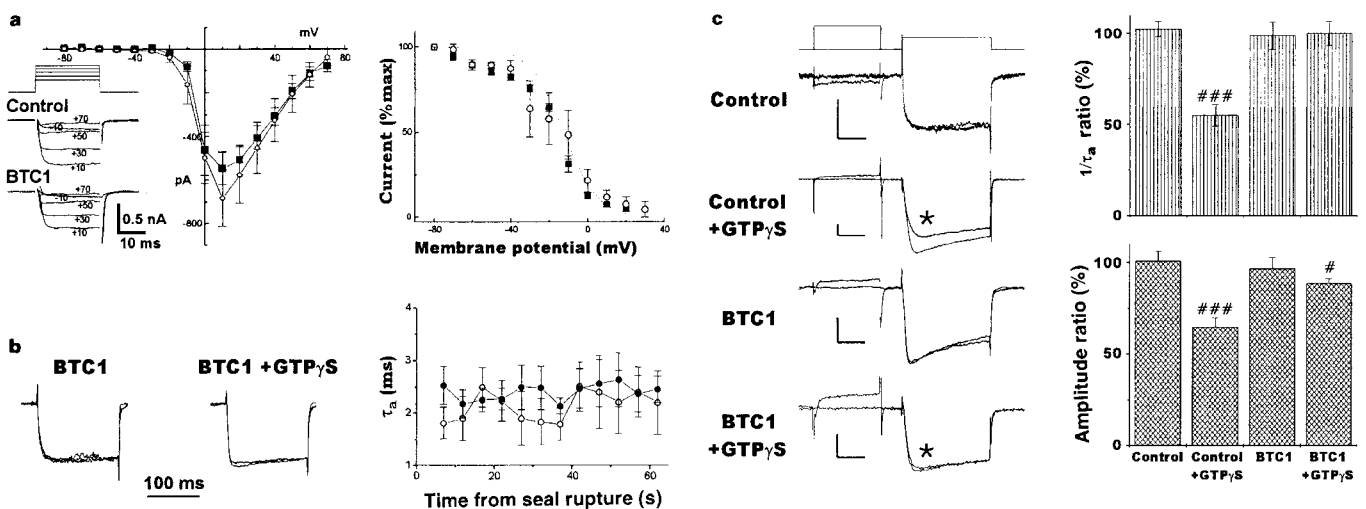


Figure 2 Effect of botulinum toxin on the calyx calcium current and its modulation by GTP- γ S. **a**, Left, leak-corrected currents evoked by step depolarizations, together with a plot of the peak current-to-voltage relation (mean $\pm$ s.e. for 6 calyces) in control ($\blacksquare$) and BTC1-treated ($\circ$) calyces. Right, calcium current steady-state inactivation curves in control ($\blacksquare$), $n = 6$, and BTC1-treated ($\circ$), $n = 6$, calyces. Pooled data were fitted by a Boltzman relation, with $V_{1/2}$ and k values of -12 and 9 mV respectively. **b**, Wash-in of GTP- γ S; conditions as for Fig. 1a. Note the constancy of the current activation rate during GTP- γ S infusion in both the superimposed current traces (left panel) and τ_a (right panel). Peak currents: 288 pA BTC1, 590 pA BTC1 + GTP- γ S. **c**, Test for reluctant calcium channels in control and BTC1-treated calyces. Left, current traces recorded

with/without a 60 ms prepulse to $+80$ mV. Note the increase in current induced by the prepulse in the control but not the BTC1 calyx with GTP- γ S (asterisks). Calibration bars: 50 ms and 200 pA. Right, histograms of percentage ratios of with prepulse to without prepulse for $1/\tau_a$ (upper histogram; with GTP- γ S, values were: control, $55 \pm 6\%$; BTC1, $100 \pm 7\%$, $n = 8$; $P_i < 0.01$) and current amplitude (lower histogram; with GTP- γ S, values were: control, $65 \pm 5\%$; BTC1 $89 \pm 6\%$, $n = 8$; $P_i < 0.01$). ###, $P_i < 0.01$ compared to either untreated controls or BTC1-treated with GTP- γ S. There was a small, but statistically significant residual G-protein effect on the calcium amplitude in BTC1-treated calyces: #, $P_i < 0.05$ compared to untreated, but not BTC1-treated, controls. Values of n : control, 5; control + GTP- γ S, 8; BTC1, 4; BTC1 + GTP- γ S, 8.

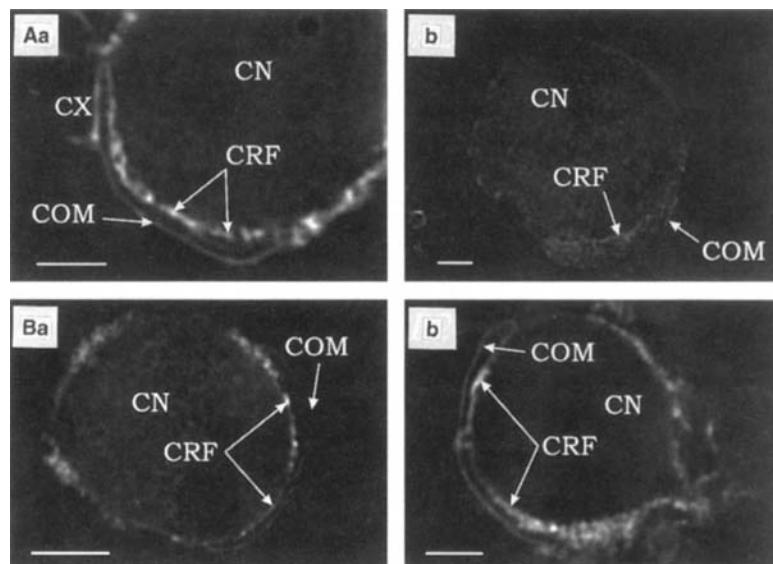


Figure 3 Effect of botulinum toxin C1 on immunofluorescence staining of release-site-associated proteins. Each panel shows a single calyx synapse stained with antibody. **A**, Anti-syntaxin staining. In controls (**a**), staining was restricted to the calyx (CX) membrane on both its inner release face (CRF) and outer membrane (COM). The calyx can be seen closely apposed to the ciliary neuron (CN). Note the patchy staining of the release face in the control calyx which may indicate release sites. In the BTC1-treated terminals (**b**) calyx membrane staining was markedly reduced, with a loss of bright patches and increased staining of the cytoplasm. The ratio of presynaptic membrane to cytoplasm staining was 3.3 ± 0.2 , $n = 10$, in controls, and 1.8 ± 0.2 , $n = 8$, in BTC1 terminals ($P_i < 0.01$). **B**, Staining of calyces with antibodies against the C-terminal domain of SNAP-25. As

with syntaxin, SNAP-25 staining was observed on both the release and non-release faces of the control terminals (**a**), with marked clustered staining of the release face. Staining of BTC1-treated calyces (**b**) was very similar. The ratio of membrane to cytoplasm staining was not significantly different when using an antibody against the C-terminal region for controls (6.0 ± 1.1 , $n = 12$) and BTC1-treated terminals (7.1 ± 1.0 , $n = 13$; $P_i > 0.1$), or an antibody against the N-terminal region (images not shown) (2.3 ± 0.2 , $n = 6$, and 2.8 ± 0.2 , $n = 6$; $P_i > 0.1$, respectively). Scale bar, 5 μ m. There was no change after BTC1 treatment in staining of calyces with a polyclonal antibody against G_{α_o} (from J. K. Northup), indicating that the toxin did not decrease G proteins.

3B and legend). No difference in staining was observed between control and BTC1-treated calyces with either antibody. Thus syntaxin is the primary target of BTC1 in the chick calyx under our conditions.

Coexpression of N-type calcium channels with syntaxin in frog oocytes has indicated that this protein has effects on the long-term kinetics of the channel, shifting the steady-state inactivation range by -10 to -20 mV (refs 26 and 27, respectively). BTC1 treatment cleaves syntaxin and would be expected to reverse this effect, but there was no significant shift in the inactivation curve (Fig. 2a, right). Thus syntaxin, or at least its BTC1-cleaved, N-terminal region, does not affect inactivation of presynaptic calcium channels. This result was not unexpected as N-type calcium channels in the nerve terminal are resistant to voltage-dependent inactivation^{7,10}. Therefore when N-type channels are inserted into the transmitter-release face some other factor seems to exert an opposite and stronger effect on inactivation than syntaxin.

The elimination of the effect of activated G proteins on the calcium current by BTC1 indicates that syntaxin plays a key role in this mechanism of modulation in the nerve terminal. BTC1 cleaves syntaxin at amino acid 253 (ref. 28), leaving only the 253/4–288 membrane-spanning stump. The cleaved N-terminal segment of syntaxin is critical for binding several presynaptic proteins²⁴. We do not know precisely the role syntaxin plays in G-protein modulation of the presynaptic calcium channel: it may form part of, or indirectly modify, the activated G-protein binding site on the calcium channel, or it may play a role in colocalizing the G protein and the channel.

Calcium channels that are attached to the transmitter release site are most likely to gate secretion^{2,29}. Regulation of the sensitivity to G proteins by linkage to syntaxin could render only the release-site-linked calcium channels susceptible to this form of modulation. Our results suggest that the properties of a channel may be

modified by its attachment to a specific cellular apparatus, so that channels associated with a particular cell function are regulated as an operational group, independent of their unmodified neighbours. □

Methods

Calyces were prepared by gentle enzymatic dissociation^{7,11}. The external recording medium was (in mM): sodium-*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES) 115, CaCl_2 10, MgCl_2 1, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) 10, tetraethylammonium chloride 10, with 1 μ M tetrodotoxin. The patch (internal) solution contained: caesium gluconate 120, CsCl 30, ethyleneglycol-bis (β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) 10, MgCl_2 3, HEPES 10, ATP 2; adjusted to pH 7.3 with CsOH. GTP- γ S (Sigma) at 0.2 mM was added to the intracellular solution and aliquots were stored at -70°C . This solution was kept on ice during the experiment. Current activation time constants (τ_a) were fitted using an iterative procedure (pClamp 6.1 software, Axon instruments) with a single exponential and straight line, a combination that gave a good visual fit for virtually all traces analysed. Leak subtraction was by a *P/N* procedure using 6–8 prepulses. Steady-state inactivation was examined by sequential ascending holding potential steps of 5 s duration. Leak pulses followed immediately after the test pulse.

BTC1 (1 μ g in 100 μ l) was injected directly into 14th-embryonic-day chick embryos. As botulinum toxins block all cholinergic transmission, the injection was judged to be effective once the normal jerky movement of the embryo was halted. Ciliary ganglia were removed, dissociated and plated. For immunostaining the preparation was fixed 16–20 h after the onset of BTC1 treatment in 2% paraformaldehyde for 45 min and was permeabilized in 0.5% polyoxyethylene-20-cetyl ether (Brij-58; Sigma) with 0.5% paraformaldehyde for 10 min. Staining was by exposure to 0.8 $\mu\text{g ml}^{-1}$ monoclonal anti-rat syntaxin (Sigma), 0.12 $\mu\text{g ml}^{-1}$ monoclonal anti-rat SNAP-25 N-terminal (WAKO) or 1:500 dilution of polyclonal anti-recombinant C-terminal SNAP-25 (from L. Williamson¹⁹) primary antibody for 16 h, followed by $\sim 15 \mu\text{g ml}^{-1}$ CY3-

conjugated secondary antibody (Jackson) for 1 h. No staining of calyces was observed with MOPC-141 or MOPC-21 (Sigma) mouse immunoglobulin primary antibody. Cells were visualized under fluorescent illumination on a Zeiss Axiophot with a $\times 63$, 1.25 NA lens. Images were acquired and analysed using a Scanalytics Cellscan system with exhaustive photon reassignment deconvolution image enhancement.

Received 23 October; accepted 26 November 1996.

1. Llinas, R. R., Steinberg, I. Z. & Walton, K. *Biophys. J.* **33**, 323–351 (1981).
2. Stanley, E. F. *Neuron* **11**, 1007–1011 (1993).
3. Yoshida, A. *et al. J. Biol. Chem.* **267**, 24925–24928 (1992).
4. Sheng, Z.-H., Rettig, J., Takahashi, M. & Catterall, W. A. *Neuron* **13**, 1303–1313 (1994).
5. Hille, B. *Trends Neurosci.* **17**, 531–535 (1994).
6. Dolphin, A. C. *Exp. Physiol.* **80**, 1–36 (1995).
7. Stanley, E. F. & Goping, G. *J. Neurosci.* **11**, 985–993 (1991).
8. O'Connor, V. M., Shamotienko, O., Grishin, E. & Betz, H. *FEBS Lett.* **326**, 255–260 (1993).
9. Stanley, E. F. *Brain Res.* **505**, 341–345 (1989).
10. Yawo, H. & Momiyama, A. *J. Physiol. (Lond.)* **460**, 153–172 (1993).
11. Stanley, E. F. *Neuron* **7**, 585–591 (1991).
12. Scott, R. H. & Dolphin, A. C. *Neurosci. Lett.* **69**, 59–64 (1986).
13. Marchetti, C. & Robello, M. *Biophys. J.* **56**, 1267–1272 (1989).
14. Grassi, F. & Lux, H.-D. *Neurosci. Lett.* **105**, 113–119 (1989).
15. Bean, B. P. *Nature* **340**, 153–156 (1989).
16. Stanley, E. F. & Cox, C. *Ann. NY Acad. Sci.* **635**, 70–79 (1991).
17. Mochida, S., Saisu, H., Kobayashi, H. & Abe, T. *Neuroscience* **65**, 905–915 (1995).
18. Rossetto, O. *et al. Nature* **372**, 415–416 (1994).
19. Williamson, L. C., Halpern, J. L., Montecucco, C., Brown, J. E. & Neale, E. A. *J. Biol. Chem.* **271**, 7694–7699 (1996).
20. Foran, P., Lawrence, G. W., Shone, C. C., Foster, K. A. & Dolly, J. O. *Biochemistry* **35**, 2630–2636 (1996).
21. Sheng, Z.-H., Rettig, L., Cook, T. & Catterall, W. A. *Nature* **379**, 451–454 (1996).
22. Blasi, J. *et al. EMBO J.* **12**, 4821–4828 (1993).
23. Shiavo, G., Rossetto, O., Benfenati, F., Poulain, B. & Montecucco, C. *Ann. NY Acad. Sci.* **710**, 65–75 (1994).
24. Kee, Y. & Scheller, R. H. *J. Neurosci.* **16**, 1975–1981 (1996).
25. Chapman, E. R., Hanson, P. I., An, S. & Jahn, R. *J. Biol. Chem.* **270**, 23667–23671 (1995).
26. Wiser, O., Bennett, M. K. & Atlas, D. *EMBO J.* **15**, 4100–4110 (1996).
27. Bezprozvanny, I., Scheller, R. H. & Tsien, R. W. *Nature* **378**, 623–626 (1995).
28. Schiavo, G., Shone, C. C., Bennett, M. K., Scheller, R. H. & Montecucco, C. *J. Biol. Chem.* **270**, 10566–10570 (1995).
29. Simon, S. M. & Llinas, R. R. *Biophys. J.* **48**, 485–498 (1985).

Acknowledgement: We thank P. S. Pennefather, H. Gainer and P. J. Church for helpful comments.

Correspondence and requests for materials should be addressed to E.F.S. (e-mail: elis@helix.nih.gov).

Regulation by insulin of a unique neuronal Ca^{2+} pool and of neuropeptide secretion

Elizabeth A. Jonas*, Ronald J. Knox*,
T. Caitlin M. Smith*, Nancy L. Wayne†,
John A. Connor‡ & Leonard K. Kaczmarek*

* Department of Pharmacology, Yale University School of Medicine,
333 Cedar Street, New Haven, Connecticut 06520, USA

† Department of Physiology, University of California School of Medicine,
Los Angeles, California 90025-1751, USA

‡ Lovelace Institute, 425 Ridgcrest Drive, Albuquerque, New Mexico 87108, USA

The insulin receptor is a tyrosine kinase receptor that is found in mammalian brain¹ and at high concentrations in the bag cell neurons of *Aplysia*². We show here that insulin causes an acute rise in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in these neurons and triggers release of neuropeptide. The insulin-sensitive intracellular Ca^{2+} pool differs pharmacologically from previously described Ca^{2+} stores that are sensitive to inositol trisphosphate and from mitochondrial Ca^{2+} stores^{3–7}. Insulin, but not thapsigargin, stimulates Ca^{2+} release at the distal tips of neurites, the presumed site of neuropeptide secretion^{8,9}. The effects of insulin on intracellular Ca^{2+} release and neuropeptide secretion occur without triggering spontaneous action potentials. The insulin-sensitive rise in $[\text{Ca}^{2+}]_i$ moves into the distal tips of neurites after exposure to a cyclic AMP analogue, a treatment that causes a similar translocation of neuronal vesicles^{10–12}. Our data indicate that

Ca^{2+} release from a distinct intracellular pool associated with secretory vesicles may contribute to secretion of neuropeptide in the absence of neuronal discharge.

Stimulation of an afferent input to the peptidergic bag cell neurons triggers repetitive firing of action potentials. This after-discharge causes the release of the neuroactive peptide egg-laying hormone (ELH)^{13,14}. Secretion can outlast the period of action potential firing¹⁵ and may therefore depend on release of Ca^{2+} from intracellular stores. We found that insulin stimulates ELH secretion without triggering action potentials. We measured ELH release while recording the electrical activity of clusters of bag cell neurons. In untreated controls, secretion of ELH could not be detected without stimulation of an afferent input^{13,14}. In contrast, treatment with insulin caused significant ELH release without electrical stimulation of the input (Fig. 1a) and did not induce spontaneous action potential firing ($n = 8$).

Direct measurements of $[\text{Ca}^{2+}]_i$ indicated that insulin causes release of Ca^{2+} from an intracellular pool. Bath application of insulin raised $[\text{Ca}^{2+}]_i$ in bag cell neurons that were injected with either Fura-2 ($n = 75$) or with Fura-2-dextran ($n = 3$), a Ca^{2+} indicator that is excluded from intracellular organelles. Normal resting $[\text{Ca}^{2+}]_i$ in isolated bag cell neurons is 150–300 nM and does not change spontaneously^{3,4}. After exposure to insulin, $[\text{Ca}^{2+}]_i$ increased within 5–20 min in 81% of neurons (Fig. 1b, c) and remained elevated for 4–24 h ($n = 10$). Insulin-induced Ca^{2+} responses of similar amplitude and kinetics were observed in the distal tips of neurites (63%, $n = 41$; Fig. 1c). The response to insulin was specific. Treatment of cells with the inactive A chain of the insulin molecule did not cause an increase in $[\text{Ca}^{2+}]_i$ (Fig. 1b).

The insulin-evoked Ca^{2+} elevation does not depend on external Ca^{2+} because insulin caused a rise in $[\text{Ca}^{2+}]_i$ in a Ca^{2+} -free EGTA solution (Fig. 1b). In another series of experiments conducted in Ca^{2+} -containing artificial sea water, external Ca^{2+} was removed after the initial response to insulin was obtained. Insulin raised $[\text{Ca}^{2+}]_i$ from 199 ± 33.5 nM to 452 ± 79.6 nM ($n = 13$, $P < 0.001$). On removal of external Ca^{2+} , $[\text{Ca}^{2+}]_i$ continued to rise (564 ± 113 nM; $P < 0.05$), indicating that the source of the increased cytosolic Ca^{2+} is intracellular (Fig. 1d).

Exposure of bag cell neurons to mammalian insulin triggers autophosphorylation on tyrosine residues of the bag cell neuron insulin receptor, an effect that is blocked by the tyrosine-kinase inhibitor herbimycin A (ref. 2). We found that pretreatment of cells with herbimycin A alone produced a small effect on $[\text{Ca}^{2+}]_i$ but significantly attenuated the response to insulin ($n = 4$; $[\text{Ca}^{2+}]_i$ in controls was 195 ± 25 nM; $[\text{Ca}^{2+}]_i$ in cells treated with herbimycin A alone was 277 ± 37 nM, $P < 0.02$; $[\text{Ca}^{2+}]_i$ in cells exposed to herbimycin A and insulin was 395 ± 113 nM; $P < 0.1$).

Stimulation of many receptors generates production of inositol trisphosphate (InsP_3), which releases intracellular Ca^{2+} in most cell types⁵, including bag cell neurons⁴. The increase in $[\text{Ca}^{2+}]_i$ in response to insulin, however, appears to be independent of InsP_3 . Microinjection of heparin, a competitive inhibitor of the InsP_3 receptor, blocks InsP_3 -mediated Ca^{2+} release¹⁶. Injection of bag cell neurons with 10–100 μM heparin blocked InsP_3 -mediated Ca^{2+} release ($n = 3$; Fig. 2) but had no effect on the subsequent ability of insulin to increase $[\text{Ca}^{2+}]_i$ (from 196 ± 37 nM to 620 ± 124 nM; $P < 0.025$). After changing to a Ca^{2+} -free medium, $[\text{Ca}^{2+}]_i$ remained elevated in the heparin-injected, insulin-treated cells. In insulin-treated cells that were not injected with heparin, InsP_3 caused a rapid elevation of $[\text{Ca}^{2+}]_i$ (Fig. 2), consistent with previous findings in untreated bag cell neurons⁴. The mean $[\text{Ca}^{2+}]_i$ in insulin-treated cells before InsP_3 injection was 605 ± 205 nM, and the mean peak $[\text{Ca}^{2+}]_i$ following InsP_3 injection was 975 ± 319 nM ($n = 5$, $P < 0.05$).

Depleting Ca^{2+} stores in the endoplasmic reticulum (ER) with thapsigargin does not prevent the action of insulin. Thapsigargin treatment of bag cell neurons depletes ER stores by inhibiting the ER

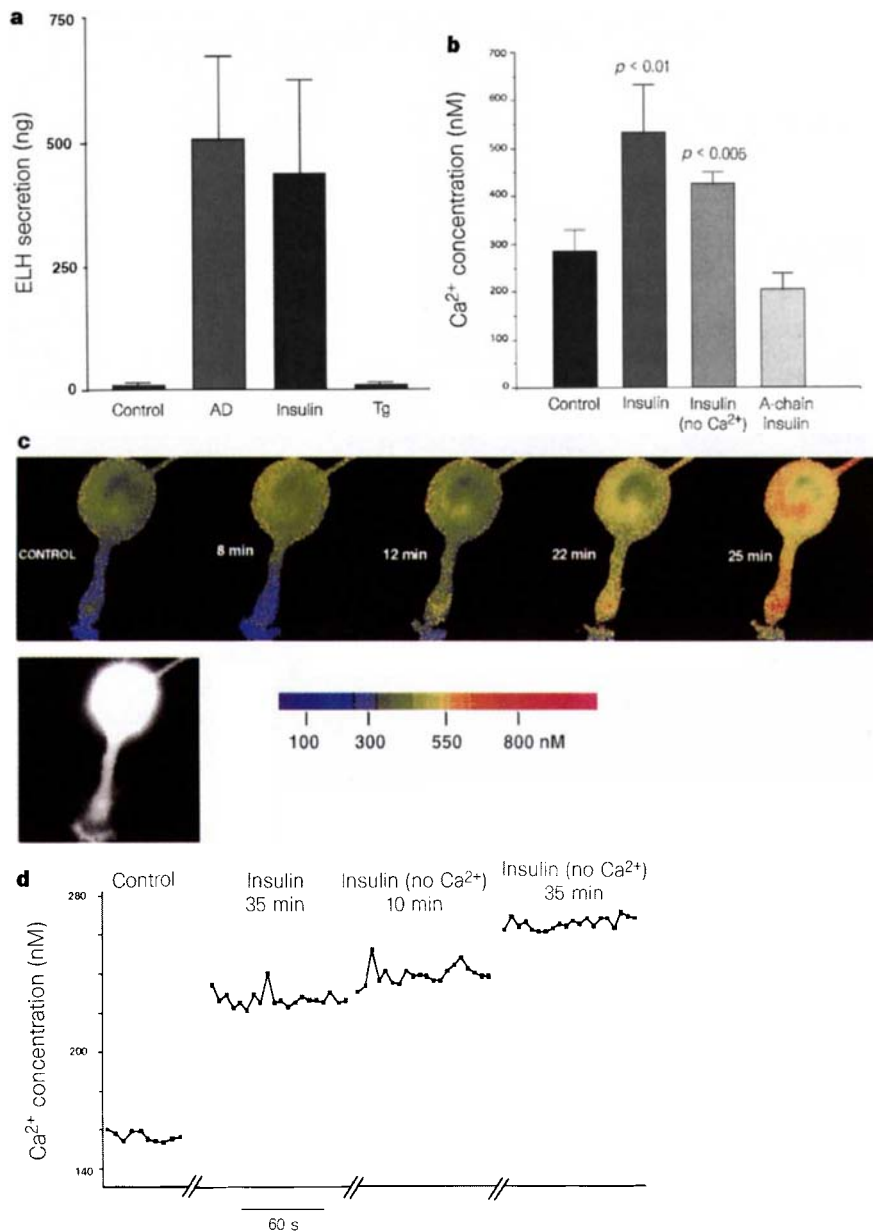


Figure 1 Insulin promotes secretion of ELH in the absence of an after-discharge and increases $[Ca^{2+}]_i$. **a**, Effects of after-discharge (AD), insulin ($5 \mu M$) and thapsigargin (Tg; $5 \mu M$) on the amount of ELH secreted from bag cell clusters. **b**, $[Ca^{2+}]_i$ levels in isolated cells exposed to insulin ($5 \mu M$) in normal artificial sea water (ASW; $n = 8$) or Ca^{2+} -free ASW ($n = 5$), or to the inactive A chain of the insulin molecule ($5 \mu M$ in ASW; $n = 2$). **c**, Time course of insulin action in a Fura-2-

injected neuron. A rise in cytosolic Ca^{2+} is indicated by an increase in the colour-coded fluorescence intensity ratio. **d**, Extracellular Ca^{2+} is not required for insulin-induced Ca^{2+} release. $[Ca^{2+}]_i$ is shown at one position on the soma of a bag cell neuron during 2-min intervals before and after insulin application in both ASW and Ca^{2+} -free ASW.

Ca^{2+} pump and preventing re-uptake of Ca^{2+} into the ER^{6,17}. Bag cell neurons pretreated with thapsigargin and subsequently exposed to insulin in a Ca^{2+} -free medium responded with a further increase in $[Ca^{2+}]_i$ both in somata and in neurites. The mean somatic $[Ca^{2+}]_i$ of thapsigargin-treated cells just before insulin application was 348 ± 40 nM, and after insulin treatment rose to 695 ± 83 nM; ($P < 0.01$, $n = 6$; Fig. 3a, c). The source of this latter Ca^{2+} was not a residual thapsigargin/InsP₃ Ca^{2+} pool because injection of InsP₃ into these neurons did not significantly raise $[Ca^{2+}]_i$. Before injection, the mean $[Ca^{2+}]_i$ in thapsigargin/insulin-treated cells was 549 ± 53 nM, whereas after injection it was 625 ± 138 nM ($P < 0.2$, $n = 4$; Fig. 3b).

Thapsigargin, which significantly increases $[Ca^{2+}]_i$ at the somata in bag cell neurons³, did not promote release of ELH (Fig. 1a). We

found that another striking difference between the thapsigargin-sensitive Ca^{2+} store and the store that was responsive to insulin is spatial distribution. In cells treated with thapsigargin alone, peak levels of Ca^{2+} were attained in the somata, with a decreasing gradient along the proximal neurite ($P < 0.005$, Fig. 3d). In contrast, Ca^{2+} levels following subsequent exposure to insulin were equally elevated in soma and neurites, indicating that the insulin-sensitive store is equally represented in both compartments.

The insulin-sensitive Ca^{2+} store that we have described appears to differ from other Ca^{2+} pools which can be distinguished by their pharmacological activators. Caffeine (10 mM) applied to bath; $n = 7$, injected cyclic ADP ribose¹⁸ ($n = 4$) or ryanodine (1 μM (ref. 19), $n = 4$) did not raise $[Ca^{2+}]_i$ in bag cell neurons. $[Ca^{2+}]_i$ levels in bag cell neurons co-injected with Fura-2 and nicotinate

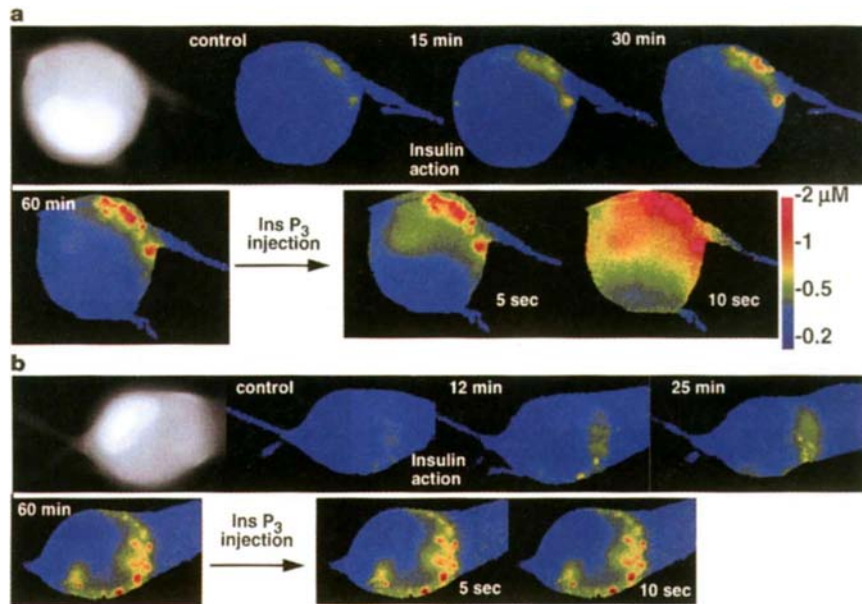


Figure 2 Insulin triggers release of intracellular Ca^{2+} from a heparin-resistant store. **a**, InsP_3 causes the release of Ca^{2+} in insulin-treated cells. On the left is shown the raw fluorescence image of the cell at an excitation wavelength of 380 nm. The following four images show the progressive increase in $[\text{Ca}^{2+}]_i$ after exposure to insulin. After switching to a Ca^{2+} -free medium, InsP_3 (5–10 μM estimated cellular concentration) was pressure-injected into the soma: the following two images show the increase in $[\text{Ca}^{2+}]_i$ at 5 and 10 s after injection of

InsP_3 . **b**, Insulin-mediated Ca^{2+} -release is not inhibited by heparin. On the left is shown the 380-nm fluorescence of a bag cell neuron preinjected with Fura-2 and heparin in a 4:1 ratio. The final concentration of heparin in the cell was calculated to be 100 μM in the soma. The next four images show that, in the presence of intracellular heparin, insulin treatment still elicited intracellular Ca^{2+} release. After 60 min, the external solution was changed to Ca^{2+} -free ASW and InsP_3 was injected into the cell. No response to InsP_3 injection could be detected (last two images).

adenine dinucleotide phosphate (NAADP; 10–50 μM final estimated intracellular concentration), an agent that releases Ca^{2+} in sea urchin eggs²⁰, were the same as those injected with Fura-2 alone ($n = 6$). Treatment with the mitochondrial uncoupling agent carbonyl cyanide *P*-(trifluoromethoxy)phenylhydrazone²¹ (FCCP; 10–20 μM), stimulated Ca^{2+} release, presumably from mitochondria. After exposure of bag cell neurons to FCCP, however, subsequent application of insulin still stimulated Ca^{2+} release (mean $[\text{Ca}^{2+}]_i$ after FCCP treatment was $337 \pm 34 \text{ nM}$, and after subsequent insulin treatment was $483 \pm 57 \text{ nM}$; $P < 0.03$, $n = 11$). Moreover, the pattern of this release was spatially distinct from that of FCCP (data not shown).

A Ca^{2+} -containing vesicle may be the source of insulin-evoked Ca^{2+} release during secretion. Electron microscopy¹⁰ and video-enhanced microscopy^{10–12} have revealed that cAMP analogues accelerate transport of ELH-containing vesicles along microtubules from the soma and the central region of growth cones into the surrounding lamellae and distal tips of neurites (Fig. 4a). Insulin alone does not cause translocation of organelles ($n = 12$, data not shown). We found, however, that in insulin-treated cells, the site of Ca^{2+} release builds up at the distal tips of neurites after treatment with 8-(4-chlorophenylthio)-adenosine-3',5'-cyclic monophosphate (pCPT-cAMP; Fig. 4b). The effect occurred in Ca^{2+} -containing ($n = 9$) and in Ca^{2+} -free media ($n = 4$). Moreover, the movement of vesicles in response to the cAMP analogue (observed by video-enhanced microscopy) correlated with translocation of the $[\text{Ca}^{2+}]_i$ response (Fig. 4a). These data indicate that a subset of vesicular organelles whose location is regulated by cAMP may release Ca^{2+} in response to insulin.

The different Ca^{2+} release patterns of thapsigargin and insulin suggest that these agents trigger release of Ca^{2+} from different intracellular compartments. Efflux from the thapsigargin-sensitive pool in bag cell neurons does not stimulate peptide secretion and causes a minimal increase in $[\text{Ca}^{2+}]_i$ in the distal tips of neurites. In contrast, insulin-stimulated intracellular Ca^{2+} mobilization is

prominent in neurites, is associated with ELH release, and can be translocated by cAMP analogues. In non-neuronal cells, spatial differences in localization of Ca^{2+} stores can influence secretion, and secretory granules usually present at release sites in these cells have been found to discharge Ca^{2+} in a cell-free preparation upon exposure to InsP_3 (ref. 22). The insulin-sensitive Ca^{2+} store in bag cell neurons, although not sensitive to InsP_3 , could represent secretory vesicles, which are prominent in the distal tips of neurites, or may represent another type of vesicle or endosome. □

Methods

Measurement of ELH secretion. Abdominal ganglia were placed in artificial sea water (ASW) (in mM) 460 NaCl; 10.4 KCl; 11 CaCl_2 ; 55 MgCl_2 ; 10 Tris-HCl; 0.001 g ml^{-1} glucose, 500 U ml^{-1} penicillin and streptomycin, pH 7.8 at 15 °C. Bag cell clusters were perfused through the abdominal artery^{16,23} at $10 \mu\text{l min}^{-1}$ with ASW, or with ASW containing 5 μM insulin or 5 μM thapsigargin. This concentration of bovine insulin (Collaborative Biomedical) activates the bag cell-neuron insulin receptor by stimulating its autophosphorylation on tyrosine residues². All solutions contained 2% Protinate (Baxter Healthcare) and a cocktail of protease inhibitors²⁴. After-discharges were stimulated electrically²⁵ and lasted for 20–30 min. Collection of perfusate and radioimmunoassay of ELH were carried out as described²⁴. The limit of detection was $1.1 \pm 0.3 \text{ ng ml}^{-1}$ (251 pM; 2 s.d. from buffer control values of 100- μl aliquots). The intra-assay coefficient of variation of quadruplicate samples containing 106 ± 7 and $1,448 \pm 56 \text{ ng ml}^{-1}$ averaged 18%, and the inter-assay coefficient of variation of these samples averaged 10%. Each experimental group represents the amount of ELH secreted over 70 min. Clusters were treated with insulin ($n = 6$) or thapsigargin ($n = 6$) for the entire 70 min or stimulated to afterdischarge ($n = 5$) at the start of the collection period.

Imaging experiments. Isolated bag cell neurons were maintained in primary culture as described². Experiments were done in ASW or Ca^{2+} -free ASW containing (in mM), 460 NaCl; 10.4 KCl; 66 MgCl_2 ; 15 HEPES; 5 glucose; 0.5 EGTA. Neurons are shown as fluorescence-ratio images generated from 340 and 380 nm excitation of Fura-2 (Molecular Probes). Injection of Fura-2 and image acquisition and processing have been described³; video-enhanced

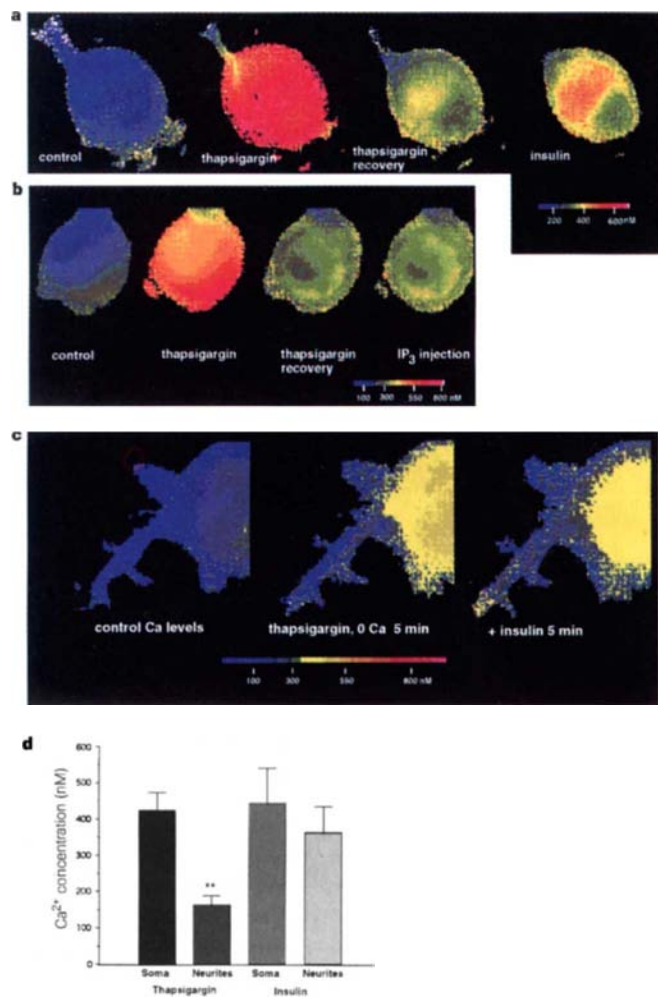


Figure 3 Depletion of ER Ca^{2+} stores with thapsigargin does not inhibit insulin-mediated Ca^{2+} release. **a**, The two left-hand images show $[Ca^{2+}]_i$ in the soma and proximal neurite of a neuron before and 20 min after thapsigargin ($7 \mu M$) was applied in Ca^{2+} -free ASW. After 40 min (third image), $[Ca^{2+}]_i$ had declined towards control levels, despite the presence of thapsigargin³. On the right the subsequent response to insulin is shown (45 min). **b**, Thapsigargin depletes $InsP_3$ -sensitive stores. The two left-hand images show $[Ca^{2+}]_i$ before and 20 min after thapsigargin exposure; the two right-hand ones show that after Ca^{2+} levels have fallen in the presence of thapsigargin, injection of $InsP_3$ fails to raise $[Ca^{2+}]_i$ (peak $InsP_3$ response shown 10 s after injection). **c**, Insulin treatment induces an increase in $[Ca^{2+}]_i$ at the tips of neurites in a Ca^{2+} -free medium. The first two panels show that thapsigargin produces a rise in $[Ca^{2+}]_i$ at the soma but little or no elevation at the neurite³. Following application of insulin (right), there was a clear increase in $[Ca^{2+}]_i$ at the tip of the neurite. **d**, Peak $[Ca^{2+}]_i$ in the soma and neurites of thapsigargin and insulin treated cells ($n = 7$). Asterisks indicate a significant difference from soma ($P < 0.005$).

microscopy was carried out according to ref. 12. Cells were observed for at least 20 min before each manipulation to verify that $[Ca^{2+}]_i$ was stable. In 8 cells, $[Ca^{2+}]_i$ was monitored for more than 1 h with no experimental manipulation in order to verify that there were no spontaneous changes in $[Ca^{2+}]_i$ over this time. Significance levels in all cases were determined by Student's *t*-test. Results are expressed as the mean $\pm$ s.e.m.

Received 14 October; accepted 29 November 1996.

- Havrankova, J., Roth, J. & Brownstein, M. *Nature* **272**, 827–829 (1978).
- Jonas, E. A., Knox, R. J., Kaczmarek, L. K., Schwartz, J. H. & Solomon, D. H. *J. Neurosci.* **16**, 1645–1658 (1996).

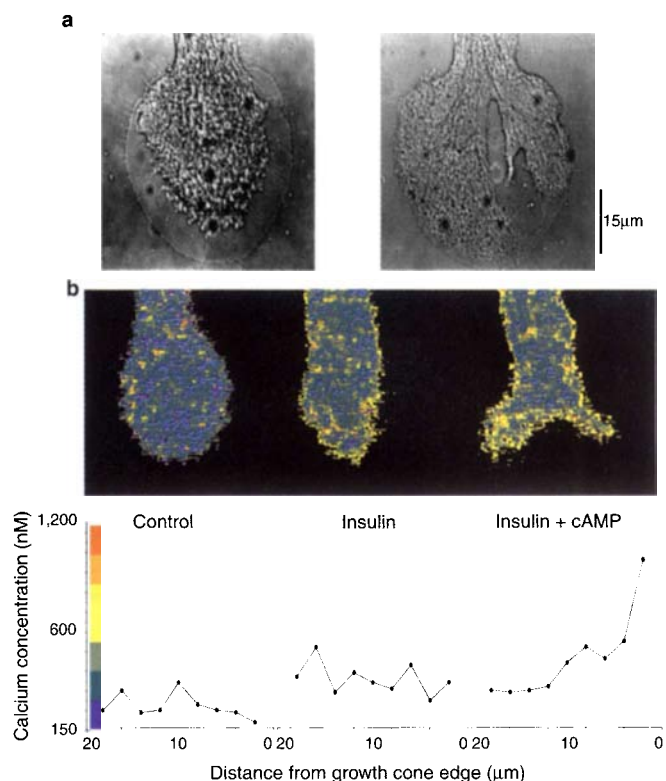


Figure 4 A cAMP analogue alters the spatial localization of insulin-sensitive Ca^{2+} release. **a**, cAMP moves vesicles into a previously vesicle-free lamellipodium. Differential interference microscopy images show a growth-cone tip before and 120 min after treatment with insulin and $200 \mu M$ pCPT-cAMP (Sigma). **b**, After insulin exposure, cAMP causes a build-up of $[Ca^{2+}]_i$ at the growth-cone tip. Images represent $[Ca^{2+}]_i$ in the neurite tip shown in **a** before, then 40 min after, insulin treatment and finally, at 80 min after exposure to pCPT-cAMP. The $[Ca^{2+}]_i$ distribution is displayed graphically below each neuron. $[Ca^{2+}]_i$ was measured within ten $4 \mu m^2$ boxes distributed along a line from the growth cone centre to its distal edge.

- Knox, R. J., Jonas, E. A., Kao, L.-S., Smith, P. J. S., Connor, J. A. & Kaczmarek, L. K. *J. Physiol.* **494**, 627–639 (1996).
- Fink, L. A., Connor, J. A. & Kaczmarek, L. K. *J. Neurosci.* **8**, 2544–2555 (1988).
- Berridge, M. J. *Nature* **361**, 315–325 (1993).
- Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R. & Dawson, A. P. *Proc. Natl Acad. Sci. USA* **87**, 2466–2470 (1990).
- Clapham, D. E. *Cell* **80**, 259–268 (1995).
- Frazier, W. T., Kandel, E. R., Kupfermann, I., Waziri, R. & Coggeshall, E. *J. Neurophysiol.* **30**, 1288–1351 (1967).
- Haskins, J. T., Price, C. H. & Blankenship, J. E. *J. Neurocytol.* **10**, 729–747 (1981).
- Forscher, P., Kaczmarek, L. K., Buchanan, J. & Smith, S. J. *J. Neurosci.* **7**, 3600–3611 (1987).
- Azhderian, E. M., Hefner, D., Lin, C.-H., Kaczmarek, L. K. & Forscher, P. *Neuron* **12**, 1223–1233 (1994).
- Knox, R. J., Quattrocki, E. A., Connor, J. A. & Kaczmarek, L. K. *Neuron* **8**, 883–889 (1992).
- Arch, S., Earley, P. & Smock, T. *J. Gen. Physiol.* **68**, 197–210 (1976).
- Stuart, D. K., Chiu, A. Y. & Strumwasser, F. *J. Neurophysiol.* **43**, 488–498 (1980).
- Wayne, N. L. & Frumovitz, M. *Endocrinology* **136**, 369–372 (1995).
- Yamamoto, H., Kanaide, H. & Nakamura, M. *Arch. Pharmacol.* **341**, 273–278 (1990).
- Lytton, J., Westlin, M. & Hanley, M. R. *J. Biol. Chem.* **266**, 17067–17071 (1991).
- Takasawa, S., Nata, K., Yonekura, H. & Okamoto, H. *Science* **259**, 370–373 (1993).
- Ehrlich, B. E., Kaftan, E., Bezprozvannaya, S. & Bezprozvanny, I. *Trends Pharmacol. Sci.* **15**, 145–149 (1994).
- Perez-Terzic, C. M., Chini, E. N., Shen, S. S., Dousa, T. P. & Clapham, D. E. *Biochem. J.* **312**, 955–959 (1995).
- Meech, R. W. & Thomas, R. C. *J. Physiol.* **298**, 111–129 (1980).
- Gerasimenko, O. V., Gerasimenko, J. V., Belan, P. V. & Petersen, O. H. *Cell* **84**, 473–480 (1996).
- Mayeri, E., Rothman, B. S., Brownell, P. H., Branton, W. D. & Padgett, L. J. *J. Neurosci.* **5**, 2060–2077 (1985).
- Wayne, N. L. & Wong, H. *Endocrinology* **134**, 1046–1054 (1994).
- Loechner, K. J., Azhderian, E. M., Dreyer, R. & Kaczmarek, L. K. *J. Neurophysiol.* **63**, 738–744 (1990).

Acknowledgements: This work was supported by NIH grants to L.K.K., N.L.W. and E.A.J. We thank D. Clapham for NAADP, B. Ehrlich for advice on experiments with ryanodine, and J. Schwartz and D. Solomon for discussion.

Correspondence and requests for materials should be addressed to E.A.J. (e-mail: kaczmarek@biomed.med.yale.edu).

Human herpesvirus KSHV encodes a constitutively active G-protein-coupled receptor linked to cell proliferation

Leandros Arvanitakis^{*}, Elizabeth Geras-Raaka[†], Anjali Varma[†], Marvin C. Gershengorn[†] & Ethel Cesarman^{*}

^{*} Department of Pathology and [†] Division of Molecular Medicine, Department of Medicine, Cornell University Medical College, 1300 York Avenue, New York, New York 10021, USA

Kaposi's sarcoma-associated herpesvirus (KSHV, also known as human herpesvirus 8, or HHV 8) is a virus that is consistently present in Kaposi's sarcoma^{1,2} and in primary-effusion (body-cavity-based) lymphomas³, malignancies that occur frequently, but not exclusively, in AIDS patients. KSHV is a gamma herpesvirus with homology to herpesvirus Saimiri and Epstein-Barr virus^{1,4}, both of which can transform lymphocytes⁵. Cloning of a KSHV genome fragment revealed the presence of an open reading frame encoding a putative G-protein-coupled receptor⁶ that is homologous to a G-protein-coupled receptor encoded by herpesvirus Saimiri^{7,8} and to human interleukin-8 receptors^{9,10}. Here we show that the KSHV G-protein-coupled receptor is a bona fide signalling receptor which has constitutive (agonist-independent) activity in the phosphoinositide-inositoltrisphosphate-protein kinase C pathway. Furthermore, the KSHV G-protein-coupled receptor stimulates cellular proliferation, making it a candidate viral oncogene.

To investigate whether the KSHV G-protein-coupled receptor (GPCR) gene encodes a functional receptor, we expressed it in COS-1 cells and assayed its binding to interleukin (IL)-8 and other chemokines. IL-8, which is a member of the family of CXC chemokines¹¹, was chosen as radioligand because of the homology of KSHV GPCR to cellular IL-8 receptors. Furthermore, as Kaposi's sarcoma lesions are sites of active inflammation and produce IL-8 and other cytokines¹², such areas may provide stimulatory signals for the KSHV GPCR. We investigated the binding characteristics of KSHV GPCR using ¹²⁵I-labelled IL-8 and various concentrations of other chemokines in a competition analysis. As shown in Fig. 1a, KSHV GPCR binds IL-8 with an apparent equilibrium dissociation constant (K_d) of 25 nM, which indicates a lower affinity than IL-8 has for the human IL-8 receptor types A or B (K_d , 0.8–1.0 nM)^{9,10}. As chemokine receptors may bind more than one ligand, we tested several other CXC chemokines, namely MGSA, NAP-2 and PF-4 (ref. 11), and found that they also bound to KSHV GPCR (Fig. 1a). Although chemokines of the CC family do not bind to IL-8 receptors¹¹, we found that I-309 and RANTES bound to KSHV GPCR, whereas MIP-1 β and MCP-1 did not (Fig. 1b). The rank order of binding was: IL-8 = NAP-2 > PF-4 = I-309 = MGSA > RANTES >> MIP-1 β and MCP-1. Thus, KSHV GPCR exhibits binding characteristics of a chemokine receptor with affinity for a range of chemokines spanning the CXC and CC families.

To determine whether KSHV GPCR is a signal-transducing receptor, we measured the formation of intracellular second messengers in COS-1 cells transiently expressing KSHV GPCR. We measured inositol phosphate (InsP) accumulation because IL-8 receptors signal through the phosphoinositide-InsP-calcium/diacylglycerol-protein kinase C cascade^{9,10}. Stimulated InsP formation

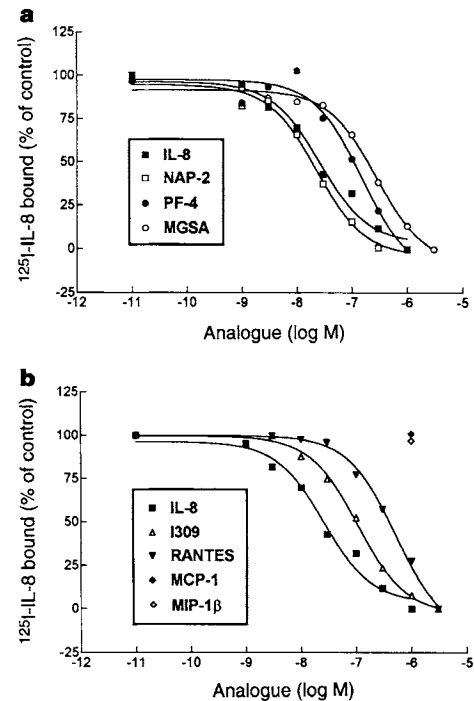


Figure 1 Competitive binding of human recombinant ¹²⁵I-labelled IL-8 by unlabeled chemokines to COS-1 cells transfected with pKSHV GPCR (10 μ g ml⁻¹). **a**, CXC chemokines (K_d or equilibrium inhibition constant, K_i): IL-8 (25 nM), NAP-2 (23 nM), PF-4 (150 nM) and MGSA (270 nM). **b**, CC chemokines (K_d or K_i): I-309 (110 nM) and RANTES (520 nM). Data represent the mean of triplicate determinations in one of two representative experiments.

can be measured in COS-1 cells coexpressing IL-8 receptors and the α subunits of the heterotrimeric proteins G₁₆ (α_{16}) or G₁₅ (α_{15}) in the presence but not in the absence of IL-8 (ref. 13). We too found no accumulation of inositol phosphates in COS-1 cells expressing IL-8 receptors in the absence or presence of IL-8, unless cotransfected with α_{16} or α_{15} (not shown) (Fig. 2a). In cells expressing IL-8 receptors and α_{16} or α_{15} , IL-8 stimulated InsP formation. In contrast, and under these experimental conditions, cells transiently expressing KSHV GPCR accumulated inositol phosphates to higher levels than those obtained with human IL-8 receptors activated by IL-8. In cells transiently expressing KSHV GPCR, InsP formation increased in the absence of added ligand and without coexpression of α_{16} or α_{15} , and was not increased by IL-8 or any of the chemokines that bound to KSHV GPCR (data not shown). The rate of InsP accumulation was constant for at least 4 hours (Fig. 2b); IL-8 had no effect at any time (data not shown). Transfection of COS-1 cells with increasing amounts of plasmid encoding KSHV GPCR showed that InsP accumulation was directly proportional to the amount of plasmid used for transfection (between 0.1 and 3 μ g ml⁻¹ plasmid) (Fig. 3). Again, there was no effect of IL-8 on InsP accumulation at any level of KSHV GPCR expression. Thus, KSHV GPCR has agonist-independent (or constitutive) activity in COS-1 cells. Although this activity was not increased by any chemokine that binds to KSHV GPCR (data not shown), it is unclear whether the constitutive activity of KSHV GPCR is maximal or whether another unidentified ligand(s) may lead to greater signalling activity. Also, and in contrast to IL-8 receptors, KSHV GPCR couples effectively to the G protein(s) endogenous in COS-1 cells. As InsP accumulation from the KSHV GPCR is resistant to pertussis toxin (data not shown), this indicates that the receptor may signal through the Gq family of G proteins.

To determine whether the constitutive signalling activity of

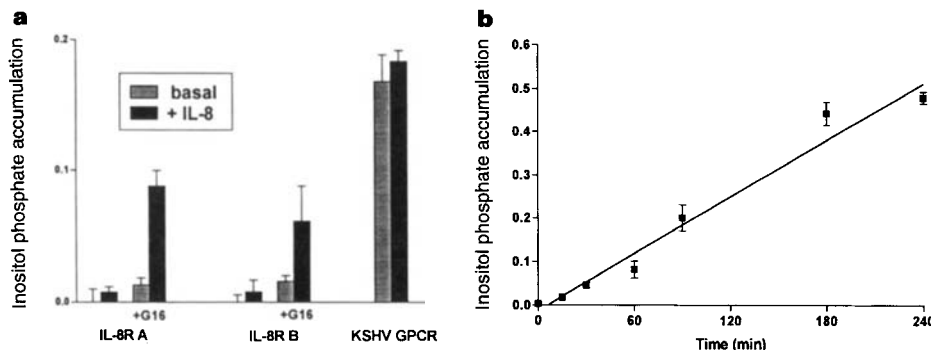


Figure 2 Inositol phosphate (InsP) accumulation in COS-1 cells transfected with pcKSHV GPCR. **a**, Comparison with COS-1 cells transfected with plasmids for human IL-8 receptors (type A or B; $10 \mu\text{g ml}^{-1}$) in the presence or absence of plasmid encoding $\text{G}\alpha_{16}$ ($2 \mu\text{g ml}^{-1}$) after 90 min. **b**, Time course in COS cells

transfected with $10 \mu\text{g ml}^{-1}$ pcKSHV GPCR. Data represent the mean $\pm$ s.d. of triplicate determinations in a representative of three experiments; InsP accumulation in mock-transfected, untreated cells has been subtracted from all points.

KSHV GPCR stimulates downstream processes, we measured the effect of basal second-messenger formation on gene transcription. We used a reporter gene, firefly luciferase, under the control of a protein kinase C (PKC)-responsive promoter containing an AP-1-binding motif¹⁴ to measure the activity of the diacylglycerol/PKC limb of the phosphoinositide signalling pathway. In these experiments, COS-1 cells were cotransfected with plasmid encoding KSHV GPCR and a plasmid containing a minimal promoter derived from the human *c-fos* gene, to which a PKC-responsive element was inserted to drive luciferase transcription¹⁴. As shown in Fig. 4, there was a direct relation between the amount of plasmid encoding KSHV GPCR used for transfection and luciferase activity. Thus, second-messenger formation caused by the basal activity of KSHV GPCR is sufficient to induce gene transcription, at least for a PKC-responsive promoter. This finding is important, given the wide range of genes, including those for inflammatory and angiogenic agents, whose transcription is regulated by PKC-responsive promoters¹⁵. Luciferase activity (Fig. 4) may be stimulated at lower plasmid levels than InsP accumulation (Fig. 3) because induction of gene transcription is an amplified response downstream of second-messenger formation.

To test whether constitutive KSHV GPCR signalling affects cellular proliferation, NRK-49F cells were transiently transfected with plasmid encoding KSHV GPCR and their proliferation was compared with cells transfected with control vector (negative control) or with a plasmid encoding the *ras* oncogene (positive control; not shown)¹⁶. Culture dishes transfected with plasmids encoding activated Ras contained more cells than control dishes

within 24–48 h. As early as 72 h post-transfection, cells transfected with plasmid encoding KSHV GPCR were increased in number compared with control transfectants, as confirmed by phase-contrast microscopy and counting after 5 days in culture (Fig. 5). All clusters transfected with plasmid encoding KSHV GPCR contained many more, closely apposed cells than did clusters of cells transfected with control plasmid.

As gamma herpesviruses cause transformation⁵ and GPCRs are oncogenic under some circumstances¹⁷, KSHV may be a transforming agent that acts, at least in part, through KSHV GPCR. This possibility is supported by the specific association of KSHV with Kaposi's sarcoma and primary effusion lymphomas, and the fact that KSHV GPCR transcripts are expressed in both types of malignancy^{6,18}. We have shown that KSHV GPCR is a bona fide receptor that signals constitutively through the phosphoinositide pathway. Although it has an amino-acid sequence that is homologous to IL-8 receptors, binds IL-8 with relatively high affinity, and activates the same signal-transduction cascade, KSHV GPCR has two features that make it a more promiscuous receptor and one more likely to be involved in pathogenesis than IL-8 receptors. KSHV GPCR signals constitutively, that is, it does not require activation by an agonist(s) and couples to a wider variety of G proteins than do IL-8 receptors. Coupling of KSHV GPCR to G proteins other than $\text{G}\alpha_{16}$, as shown by signalling in COS-1 cells that do not express this G protein, would allow KSHV GPCR to signal in a broader range of cell types. Constitutive signalling, as shown by some native GPCRs¹⁹, occurs more commonly with mutated GPCRs^{20,21} in many human diseases and in tumours^{22,23}. The

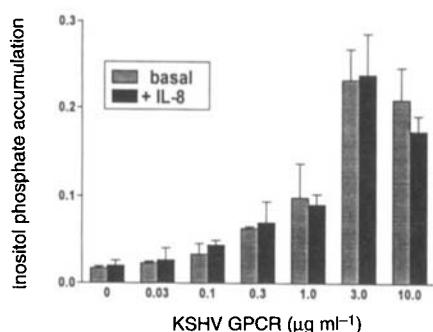


Figure 3 Dose dependence of inositol phosphate accumulation in COS-1 cells after transfection with various amounts of pcKSHV GPCR (from 0.003 to $10 \mu\text{g ml}^{-1}$) at 90 min. Data represent the mean $\pm$ s.d. of triplicate determinations in a representative of three experiments.

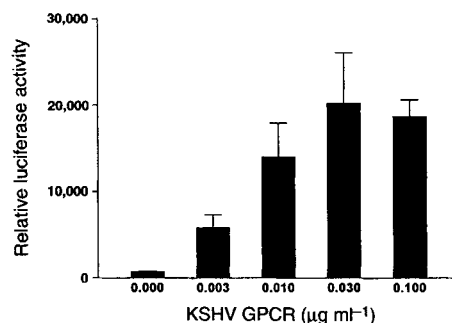


Figure 4 Transcription of luciferase driven by a PKC-responsive promoter in COS-1 cells transfected with pcKSHV GPCR. Data represent the mean $\pm$ s.d. of triplicate determinations in a representative of three experiments.

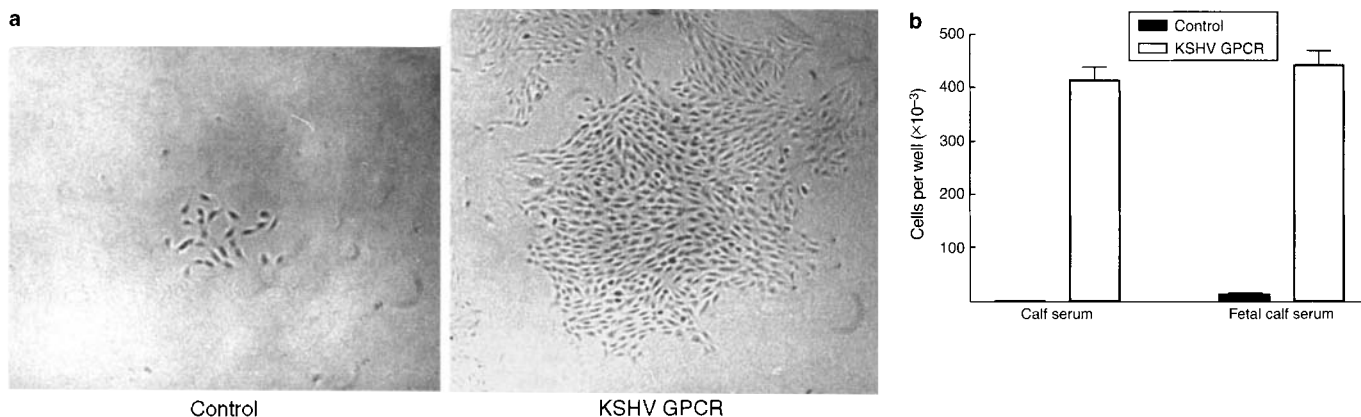


Figure 5 Cellular proliferation in NRK-49F cells transfected with control plasmid or pcKSHV GPCR. After 5 days in culture in medium supplemented with calf serum or fetal calf serum, transfected cells were trypsinized and counted. **a**,

Micrographs of representative cell clusters. **b**, Cell counts; data represent the mean $\pm$ s.d. of triplicate culture dishes of three transfections.

enhanced proliferation of KSHV GPCR transfectants supports a role for this receptor in tumorigenesis.

The presence of this functional, constitutively active GPCR in the genome of a virus that is tightly associated with specific malignancies provides circumstantial evidence for its pathogenic role. There are two possibly complementary ways in which viral GPCR acts in the development of KSHV-associated diseases: directly with constitutive signalling of KSHV GPCR leading to altered growth and neoplastic transformation, and/or indirectly, whereby signalling by KSHV GPCR induces the expression of other genes which are in turn involved in pathogenic events. The indirect mechanism might apply to basic fibroblast growth factor for example, which is pivotal in the development of Kaposi's sarcoma lesions²⁴ and whose expression is driven by an AP-1-binding, and probably PKC-responsive, promoter²⁵. Further investigation should clarify the role played by this viral gene in KSHV-associated disorders. □

Methods

Competition binding. The coding sequence of KSHV GPCR was cleaved from its original subcloning plasmid⁶ by digestion with *NcoI* and inserted into the *EcoRV* site of the eukaryotic expression vector pcDNA3.1(+) (Invitrogen), producing the pcKSHV GPCR used for expression in mammalian cells. COS-1 cells (American Type Culture Collection (ATCC)) were grown in 100-mm Petri dishes in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) with 5% Nu-Serum (Collaborative Research) in a humidified incubator in an atmosphere of 5% CO₂ at 37°C. DEAE-dextran transfections were carried out as described²⁶. COS-1 cells were transfected with 10 μ g ml⁻¹ pcKSHV GPCR and tested for IL-8 binding 48 h post-transfection. About 100,000 cells per well (in 12-well plates) were washed with Hank's balanced salt solution (HBSS) containing 25 mM HEPES, pH 7.4 (Gibco BRL). Between 0.3 and 0.5 nM [¹²⁵I]-IL-8 (DuPont-NEN) in the absence or presence of various concentrations of unlabelled chemokines was added and binding continued at 4°C for 2 h with gentle rocking. The binding buffer contained BSA at 1 mg ml⁻¹, bacitracin (1 mg ml⁻¹), and PMSF at 1 mM. Assays were terminated by aspirating the binding buffer, washing three times with chilled HBSS and solubilizing the cells with 0.4 M NaOH. An aliquot of this solution was counted in a gamma counter and data were expressed as a percentage of maximum bound. Binding and dose-response curves were analysed using Prism software (GraphPad).

Inositol phosphate accumulation. COS-1 cells were transfected with pcKSHV GPCR or plasmids encoding human IL-8 receptor types A or B and either G α_{16} or G α_{15} (not shown)¹³. All transfection cocktails were supplemented to contain equal total plasmid DNA by addition of appropriate amounts of vector (pcDNA3.1). 24 h after transfection, cells were collected and reseeded in 12-well plates in DMEM with 10% Nu serum and 1 μ Ci ml⁻¹ myo-[³H]inositol (DuPont-NEN), and maintained in culture as before for an additional 24 h. After removing the culture medium and rinsing the cells, cells were

incubated in HBSS containing 25 mM HEPES, pH 7.4, with 10 mM LiCl in the absence or presence of human recombinant IL-8 (R&D Systems) at 0.25 μ g ml⁻¹. Cells were collected after 90 min or at other times and accumulated InsP measured using ion-exchange chromatography as described²⁷. Inositol phosphate accumulation was calculated as [³H]InsPs/[³H]lipids + [³H]InsPs.

Luciferase assay. Cells were transfected with increasing amounts of pcKSHV-GPCR (0.003 to 0.100 μ g ml⁻¹) and 5 μ g ml⁻¹ pAPI-fos-Luc¹⁴, supplemented with vector (pcDNA3.1) to contain equal amounts of total plasmid DNA. Cells were washed with PBS and lysed with 0.5 ml per 10-cm dish of lysis buffer (25 mM Gly-Gly, 15 mM MgSO₄, 4 mM EGTA, 1 mM DTT, 1% Triton X-100, pH 7.8). Cell extracts were diluted 1:10 or 1:20 in reaction buffer (25 mM Gly-Gly, 15 mM MgSO₄, 4 mM EGTA, 1 mM DTT, 15 mM KH₂PO₄, 2 mM ATP, pH 7.8), then combined with equal volumes of 0.4 mM luciferin in reaction buffer, and luminescence was measured for 10 s.

Cell-proliferation assay. Normal rat kidney fibroblasts (NRK-49F; ATCC) were seeded at 2 $\times$ 10⁵ per 100-mm dish, grown in DMEM with 10% FCS, and transfected with control vector (20 μ g per plate), pcKSHV GPCR (20 μ g per plate) or pRas-Zip (10 μ g per plate; positive control; not shown)¹⁶ using calcium phosphate as described²⁸. Cells were collected 24 h post-transfection and seeded at a density of 5,000 cells per 100-mm dish in DMEM supplemented with either 10% calf serum or FCS. On day 5 post-transfection, cells were photographed for qualitative assessment (Nikon inverted phase microscope) and then trypsinized to determine total cell number using a haemocytometer.

Received 19 September; accepted 22 November 1996.

1. Chang, Y. *et al.* *Science* **266**, 1865–1869 (1994).
2. Moore, P. S. & Chang, Y. *N. Engl. J. Med.* **332**, 1181–1185 (1995).
3. Cesarman, E., Chang, Y., Moore, P. S., Said, J. W. & Knowles, D. M. *N. Engl. J. Med.* **332**, 1186–1191 (1995).
4. Moore, P. S. *et al.* *J. Virol.* **70**, 549–558 (1996).
5. Roizman, B. in *Fields Virology* (eds Fields, B. N., Knipe, D. M. & Howley, P. M.) 2221–2231 (Lippincott-Raven, Philadelphia, 1996).
6. Cesarman, E. *et al.* *J. Virol.* **70**, 8218–8223 (1996).
7. Nicholas, J., Cameron, K. R. & Honess, R. W. *Nature* **355**, 362–365 (1992).
8. Ahuja, S. K. & Murphy, P. M. *J. Biol. Chem.* **268**, 20691–20694 (1993).
9. Holmes, W. E., Lee, J., Kuang, W. J., Rice, G. C. & Wood, W. I. *Science* **253**, 1278–1280 (1991).
10. Murphy, P. M. & Tiffany, H. L. *Science* **253**, 1280–1283 (1991).
11. Baggiolini, M., Dewald, B. & Moser, B. *Adv. Immunol.* **55**, 97–179 (1994).
12. Sciacca, F. L. *et al.* *J. Immunol.* **153**, 4816–4825 (1994).
13. Wu, D., LaRosa, G. J. & Simon, M. I. *Science* **261**, 101–103 (1993).
14. Schadow, V., Barzilai, N. & Deutsch, P. *Mol. Biol. Cell* **3**, 941–951 (1992).
15. Karin, M. *J. Biol. Chem.* **270**, 16483–16486 (1995).
16. Dotto, G. P., Parada, L. F. & Weinberg, R. A. *Nature* **318**, 472–475 (1985).
17. Julius, D., Livelli, T. J., Jessell, T. M. & Axel, R. *Science* **244**, 1057–1062 (1989).
18. Arvanitakis, L. *et al.* *Blood* **88**, 2648–2654 (1996).
19. Milano, C. A. *et al.* *Science* **264**, 582–586 (1994).
20. Lefkowitz, R., Cotecchia, S., Samama, P. & Costa, T. *Trends Pharmacol. Sci.* **14**, 303–307 (1993).
21. Alblas, J., van Etten, I. & Moolenaar, W. H. *EMBO J.* **15**, 3351–3360 (1996).
22. Coughlin, S. R. *Curr. Opin. Cell Biol.* **6**, 191–197 (1994).
23. Sande, J. V. *et al.* *J. Clin. Endocrinol. Metab.* **80**, 2577–2585 (1995).
24. Ensoli, B. *et al.* *Science* **243**, 223–226 (1989).

25. Erdos, G., Lee, Y. J., Cho, J. M. & Corry, P. M. *J. Cell Physiol.* **164**, 404–413 (1995).
26. Nussenzweig, D., Thaw, C. & Gershengorn, M. *J. Biol. Chem.* **269**, 28123–28129 (1994).
27. Straub, R., Frech, G., Joho, R. & Gershengorn, M. *Proc. Natl Acad. Sci. USA* **87**, 9514–9518 (1990).
28. Loyer, A., Scangos, G. A. & Ruddle, F. H. *Proc. Natl Acad. Sci. USA* **79**, 422–426 (1982).

Acknowledgements: We thank M. Simon for plasmids encoding $G\alpha_{15}$ and $G\alpha_{16}$ cDNAs; L. Wu for plasmids encoding human IL-8 receptors, types A and B; P. Deutsch for the pAP1-fos-LUC construct; M. Gaboli for the plasmid encoding Ras; I. Clark-Lewis for the CXCL and CC chemokines; D. Fischman for use of the phase-contrast microscope; and the staff of the Division of Molecular Medicine for their generous assistance. These studies were supported by grants from the NIH to M.C.G. and to E.C.

Correspondence and requests for materials should be addressed to M.C.G. (e-mail: mcgersh@mail.med.cornell.edu).

Stress-signalling kinase Sek1 protects thymocytes from apoptosis mediated by CD95 and CD3

Hiroshi Nishina*, Klaus D. Fischer††, Laszlo Radvanyi§, Arda Shahinian*, Razqallah Hakem*, Elizabeth A. Rubie§, Alan Bernstein†, Tak W. Mak*, James R. Woodgett§ & Josef M. Penninger*

* The Amgen Institute, Ontario Cancer Institute, and Departments of Medical Biophysics and Immunology, University of Toronto, 620 University Avenue, Suite 706, Toronto, Ontario M5G 2C1, Canada

† Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario M5G 2C1, Canada

‡ Institute for Radiation and Cell Research, University of Wuerzburg, Versbacher Strasse 5, D-97078 Wuerzburg, Germany

§ Ontario Cancer Institute, Departments of Medical Biophysics and Immunology, University of Toronto, Toronto, Ontario M5G 2C1, Canada

Distinct and evolutionarily conserved signal transduction cascades mediate survival or death in response to developmental and environmental cues. The stress-activated protein kinases, or Jun N-terminal kinases (SAPKs/JNKs)^{1,2}, are activated in response to a variety of cellular stresses such as changes in osmolarity and metabolism, DNA damage, heat shock, ischaemia, or inflammatory cytokines^{3–6}. Sek1 (JNKK/MKK4) is a direct activator of SAPKs/JNKs in response to environmental stresses or mitogenic factors^{7–9}. Here we investigate the role of Sek1 in development and apoptosis by deleting *sek1* in embryonic stem (ES) cells by homologous recombination. We provide genetic evidence that different stresses utilize distinct signalling pathways for SAPK/JNK activation. *sek1*^{-/-}/*rag2*^{-/-} chimaeric mice have normal numbers of mature T cells but fewer immature CD4⁺CD8⁺ thymocytes. The *sek1* mutation did not affect the induction of apoptosis in response to environmental stresses in ES and T cells: instead, *sek1* protected thymocytes from CD95 (Fas)- and CD3-mediated apoptosis. These data indicate that SEK1 mediates survival signals in T-cell development.

SAPKs/JNKs are activated by the phosphorylation of tyrosine and threonine residues, which is catalysed by the dual-specificity kinase Sek1 (MKK4 or JNKK)^{7–9}. To examine the function of the Sek1-regulated stress signalling pathway, we generated mouse ES cell lines lacking *sek1* expression by using homologous recombination (Fig. 1a). Mutations of both *sek1* alleles (*sek1*^{-/-}) were established from *sek1*^{+/-} ES cells by selection in a high dose of G418 (Fig. 1b). *sek1* messenger RNA was expressed in *sek1*^{+/-} and *sek1*^{+/-} ES cells in a manner dependent on gene dosage, but was absent in *sek1*^{-/-} ES cells (Fig. 1c). Moreover, Sek1 protein was absent in *sek1*^{-/-} ES cells (Fig. 1d).

Sek1 is a potent activator of SAPKs/JNKs (refs 7–9). To determine whether deletion of Sek1 in ES cells prevented SAPK/JNK activation, *sek1*^{+/-}, *sek1*^{+/-} and *sek1*^{-/-} ES cells were treated

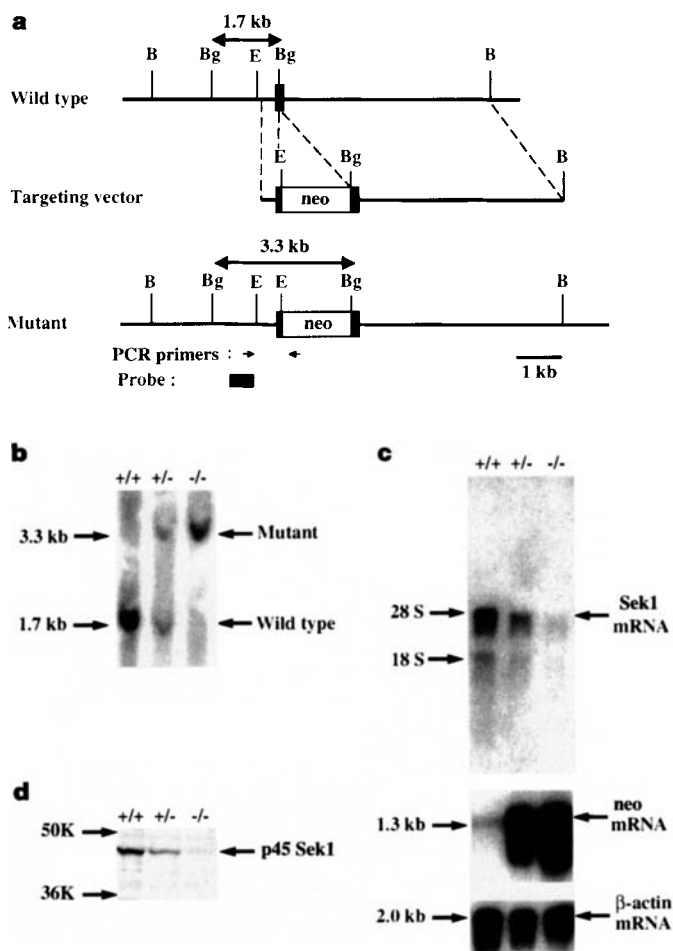


Figure 1 Gene targeting of *sek1*. **a**, Restriction map of genomic *sek1* sequences and construction of the neomycin-resistance (*neo*) insertion vector. The *sek1* exon is shown as a filled box. The *sek1* flanking probe used for Southern blotting, the expected fragment sizes after *Bgl*II digestion of wild-type (1.7 kb) and mutant (3.3 kb) genomic DNA, and the location of PCR primers are indicated. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI. **b**, Genomic analysis of embryonic stem cells. Genomic DNA was isolated from *sek1*^{+/-} (+/+), *sek1*^{+/-} (+/-) and *sek1*^{-/-} (-/-) ES cells digested with *Bgl*II and analysed by Southern blotting using the 5' flanking probe shown in **a**. Relative molecular mass markers and wild-type and mutant bands are indicated. **c**, Expression of *sek1* mRNA in *sek1*^{+/-} (+/+), *sek1*^{+/-} (+/-) and *sek1*^{-/-} (-/-) ES cells. Poly(A)-selected RNA (10 µg) was probed for *Sek1* and neomycin (*neo*) expression. β -actin is shown as a control. The 28S and 18S rRNA bands and the sizes of *sek1*, *neo* and β -actin mRNA are indicated. *sek1* mRNA was also undetectable when specific PCR primers were used with poly(A)-selected RNA from *sek1*^{-/-} ES cells (not shown). **d**, Absence of *sek1* protein in *sek1*^{-/-} ES cells. Total cell lysates from *sek1*^{+/-}, *sek1*^{+/-} and *sek1*^{-/-} ES cells (1×10^6) were probed for *sek1* protein by immunoblotting.

with anisomycin, sorbitol, ultraviolet (UV) radiation or heat shock². Whereas SAPK/JNK was activated in *sek1*^{+/-} and *sek1*^{+/-} ES cells in response to heat shock and the protein synthesis inhibitor anisomycin, these effects were abolished in *sek1*^{-/-} ES cells (Fig. 2a). Surprisingly, UV irradiation and sorbitol-induced osmolarity changes continued to activate SAPKs/JNKs to normal levels in *sek1*^{-/-} ES cells, comparable to the activation in *sek1*^{+/-} and *sek1*^{+/-} ES cells. Activation of p38/Mpk2/CSBP (refs 9, 10) in *sek1*^{-/-} ES cells was normal in response to anisomycin and sorbitol (Fig. 2b), indicating that Sek1 does not control p38 activation in response to these stresses. Induction of MAPKs by phorbol myristyl acetate (PMA) and Ca^{2+} ionophore was also unaffected in *sek1*^{-/-} ES cells (Fig. 2c). Although several SAPK/JNK activators have been

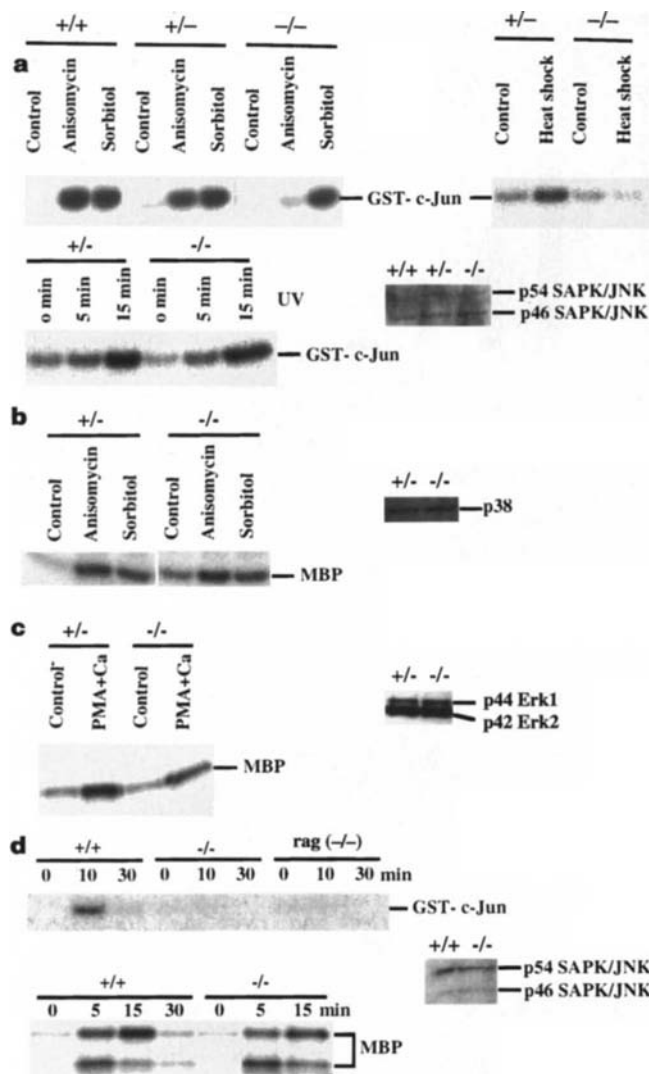


Figure 2 Activation of SAPK/JNK, p38 and MAPK in *sek1*^{-/-} ES cells and thymocytes. **a**, SAPK activity in ES cells. *sek1*^{+/+} (*+/+*), *sek1*^{+/-} (*+/-*) and *sek1*^{-/-} (*-/-*) ES cells were either untreated or activated with anisomycin, sorbitol, UV irradiation or heat shock. SAPK/JNK was immunoprecipitated and assayed for *in vitro* kinase activity using GST-c-Jun as substrate. Lower right, control western blot for p46 and p54 isoforms of SAPK/JNK in *sek1*^{+/+}, *sek1*^{+/-} and *sek1*^{-/-} ES cells. One result representative of 6 independent experiments is shown. **b**, p38/Mpk2/CSBP activity in ES cells. *sek1*^{+/+} (*+/+*) and *sek1*^{-/-} (*-/-*) ES cells were activated with sorbitol and anisomycin, and p38 kinase activity was assayed using myelin basic protein (MBP) as substrate. Right panel, western blot to control for p38 protein expression. **c**, MAPK activity in ES cells. *sek1*^{+/+} (*+/+*) and *sek1*^{-/-} (*-/-*) ES cells were activated with PMA and Ca²⁺ ionophore for 15 min, and MAPK kinase activity was assayed using MBP as substrate. A control western blot for p42 Erk2 and p44 Erk1 is shown. **d**, Activation of SAPK/JNK (upper panel) and MAPK (lower panel) activity in thymocytes. *sek1*^{+/+} (*+/+*), *sek1*^{-/-} (*-/-*) and *rag2*^{-/-} thymocytes were activated with PMA plus Ca²⁺ ionophore for 0, 5, 10, 15 and 30 min as indicated. A control western blot for SAPK/JNK isoform expression in *sek1*^{+/+} and *sek1*^{-/-} chimaeric thymocytes is shown.

obtained from chromatographically fractionated extracts^{11,12}, our results provide genetic evidence for Sek1-dependent and Sek1-independent intracellular signalling pathways for SAPK/JNK activation and show that different stresses utilize distinct signalling pathways for SAPK/JNK activation.

To determine the role of Sek1 in T-cell development, we generated *sek1*^{-/-} somatic chimaeras by Rag2 blastocyst complementation¹³.

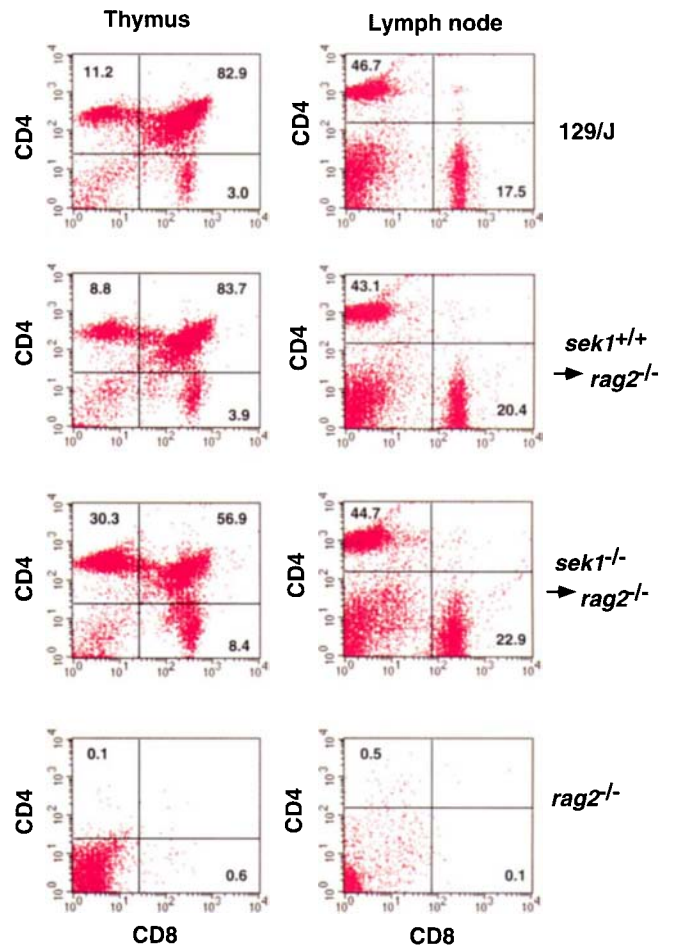


Figure 3 Immunofluorescence analysis of thymocytes (left panels) and lymph node T cells (right panels) in 129/J, *sek1*^{+/+} chimaeric, *sek1*^{-/-} chimaeric and *rag2*^{-/-} mice. Cells were isolated from 6-week-old mice and stained with anti-CD4 and anti-CD8. Percentages of positive cells within a quadrant are indicated. Total cell numbers were: 129/J thymus: 8.8×10^7 ; 129/J lymph nodes (LN): 2.2×10^7 ; *sek1*^{+/+} chimaeric thymus: 7.6×10^7 ; *sek1*^{+/+} chimaeric LN: 2.4×10^7 ; *sek1*^{-/-} chimaeric thymus: 1.1×10^7 ; *sek1*^{-/-} chimaeric LN: 1.6×10^7 ; *rag2*^{-/-} thymus: 1.1×10^6 ; *rag2*^{-/-} LN: 4×10^5 .

rag2^{-/-} mice have a block in the differentiation of their CD4⁺CD8⁻TCR⁻ thymocyte precursors to CD4⁺CD8⁺TCR⁺ immature thymocytes owing to impaired V(D)J recombination. Two independently established *sek1*^{-/-} ES cell clones (numbers 1–6 and 1–21) and parental *sek1*^{+/+} ES cells were injected into *rag2*^{-/-} blastocysts. As E14K ES cells are derived from a 129/J mouse background, age-matched 129/J mice were used as wild-type controls. *sek1*^{-/-} ES cells were able to reconstitute the T-cell compartment of *rag2*^{-/-} mice at high frequency (~70% of the mice analysed were chimaeric), and lymph nodes and spleens of *sek1*^{-/-} chimaeras had normal numbers and ratios of CD4⁺ and CD8⁺ TCRαβ⁺ T cells (Fig. 3 and Table 1).

The thymuses of *sek1*^{-/-} chimaeric mice were 4–5 times smaller than those of age-matched 129/J mice or *sek1*^{+/+} chimaeras (Table 1). The reduction in thymus size was due to a significant decrease in the population of CD4⁺CD8⁺ double-positive immature thymocytes, and a relative (but not absolute) expansion of mature CD4⁺ and CD8⁺ single-positive thymocytes (Fig. 3). Expression of the TCRαβ/CD3 complex and of thymocyte maturation markers such as CD69, CD44, HSA, CD5 and H2-K^b was comparable in *sek1*^{-/-} chimaeric, *sek1*^{-/-} chimaeric and 129/J control mice (not shown). The activation of MAPKs was similar in *sek1*^{-/-} and *sek1*^{+/+}

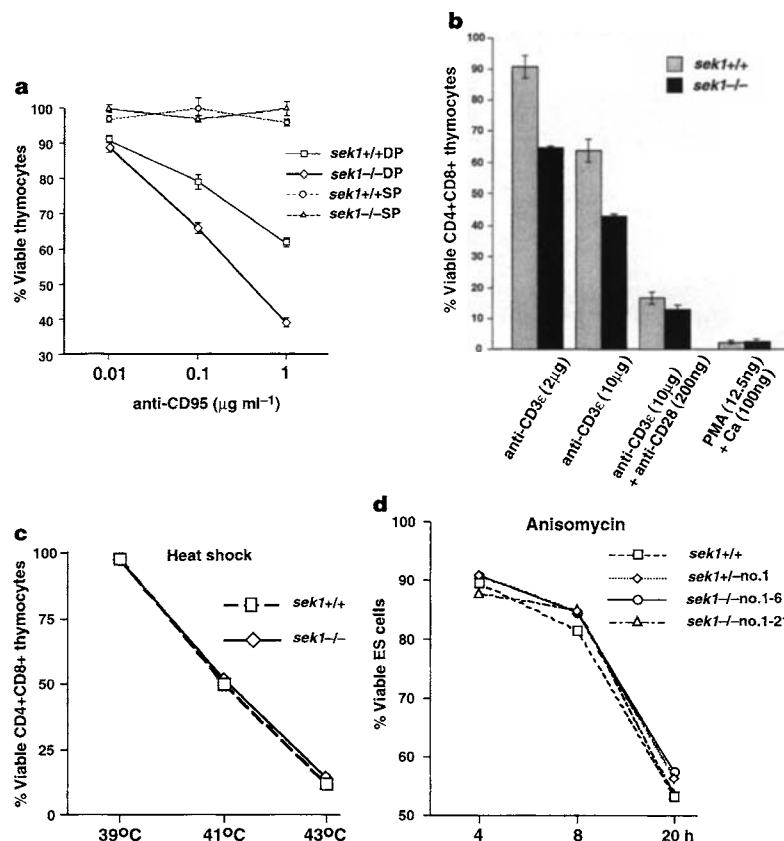


Figure 4 Induction of cell death in *sek1*^{+/+} and *sek1*^{-/-} thymocytes in response to **a**, anti-CD95; **b**, anti-CD3 ϵ ; **c**, heat shock; and **d**, induction of apoptosis in ES cells in response to anisomycin. *sek1*^{+/+} DP and *sek1*^{-/-} DP are CD4⁺CD8⁺ double-positive thymocytes; *sek1*^{+/+} SP and *sek1*^{-/-} SP are CD4⁺CD8⁻ or CD4⁻CD8⁺ single-positive thymocytes. SP thymocytes are shown as controls because only DP cells die in response to CD95 activation¹⁶. One result of a triplicate culture ($\pm$ s.d.) representative of 8 independent experiments is shown for each activation.

thymocytes, but SAPK/JNK was activated only in *sek1*^{+/+} and not in *sek1*^{-/-} thymocytes (Fig. 2d). All chimaeric mice appeared to be healthy and had no histological signs of defects or inflammation in any organ.

It has been proposed from transfection studies with dominant-negative signalling mutants that the Sek1 $\rightarrow$ SAPK/JNK signalling cascade is a common pathway required for the induction of apoptosis in response to many types of cellular stresses^{4-6,14,15}. To test the role of Sek1 in apoptosis of CD4⁺CD8⁺ thymocytes in response to physiological stimuli involved in selection and activation, thymocyte death was induced with CD95 (Fas; ref. 16) or CD3 ϵ crosslinking¹⁷. Although thymocytes from *sek1*^{-/-} and *sek1*^{+/+} mice expressed CD95 to the same extent on the cell surface (not shown), CD4⁺CD8⁺ thymocytes from *sek1*^{-/-} chimaeric mice were significantly more susceptible to dose-dependent CD95-mediated cell death than wild-type cells (Fig. 4a). *In vitro* CD3 ϵ crosslinking, which by itself is a poor inducer of apoptosis of immature thymocytes, also enhanced cell death of CD4⁺CD8⁺ thymocytes in the absence of Sek1 expression (Fig. 4b). Similarly, peripheral T cells from *sek1*^{-/-} mice showed increased susceptibility to CD95- and CD3-mediated apoptosis (not shown). However, there was no significant difference between *sek1*^{-/-}, *sek1*^{+/-} and *sek1*^{+/+} ES cells, thymocytes and peripheral T cells in either the extent or kinetics of cell death in response to γ -irradiation, UV irradiation, sorbitol, serum deprivation, anisomycin, heat shock, dexamethasone, anti-CD3 ϵ plus anti-CD28 crosslinking, PMA plus Ca²⁺ ionophore, or the anticancer drugs cisplatin, adriamycin or etoposide (Fig. 4c, d, and results not shown). These data show that Sek1 protects T cells from CD95- and CD3 ϵ -mediated cell death.

Distinct and evolutionarily conserved signal transduction cascades mediate survival or death in response to developmental and environmental cues. As Sek1 and SAPKs/JNKs have been implicated as a common pathway required for the induction of apoptosis^{4-6,14,15}, the SAPK/JNK signalling cascade was a prime candidate for the induction of cell death in T lymphocytes. Our data show that *sek1*^{-/-} T cells are more susceptible to apoptosis in response to the physiological stimuli CD3/TCR and CD95. CD3/TCR-mediated apoptosis of double-positive thymocytes is an important trigger of negative T-cell selection¹⁷ and CD3- and CD95-mediated cell death of activated peripheral T cells is crucial for maintenance of T-cell homeostasis and immunological tolerance¹⁸. Sek1 might also play a role in the expansion of CD4⁻CD8⁻ progenitors into CD4⁺CD8⁺ cells, which could contribute to the reduction in thymocyte numbers. Our results indicate that Sek1 mediates cell-survival signals during the development and activation of T lymphocytes. □

Methods

Gene targeting. An 8-kb genomic *sek1* fragment was isolated from a genomic 129/J library. Twelve nucleotides within exon 2 of the *sek1* gene were replaced by a neomycin (*neo*) gene cassette containing a poly(A) termination signal inserted in the sense orientation. The linearized construct was introduced into E14K ES cells by electroporation. G418-resistant ES colonies were screened for homologous recombination by PCR and Southern blotting. The frequency of homologous recombination was ~ 1 in 800. For the generation of *sek1*^{-/-} ES cell lines, G418-resistant *sek1*^{+/-} ES clones were cultured at increasing concentrations of G418 (2 mg ml⁻¹). For northern blotting, 10 μg poly(A)-selected RNA was probed with sequences specific for *sek1* (full-length *sek1*

Table 1 Numbers of lymphoid cells in *sek1*^{-/-} chimaeric mice

Lymphatic organ	Total number of cells (× 10 ⁶) ± s.e.m.			
	129/J	<i>sek1</i> ^{+/+}	<i>sek1</i> ^{-/-}	<i>rag2</i> ^{-/-}
Thymus	80 ± 6.3	85 ± 10.1	14 ± 4.1	1.1 ± 0.2
Lymph nodes	24 ± 2.2	22 ± 4.6	29 ± 4.2	0.7 ± 0.2
Spleen	21 ± 3.4	32 ± 3.1	27 ± 5.3	8.3 ± 1.6

Total thymocytes, total mesenteric lymph-node cells and total spleen cells from 129/J (*n* = 6), *sek1*^{+/+} chimaeric (*n* = 3), *sek1*^{-/-} chimaeric (*n* = 6), and *rag2*^{-/-} (*n* = 5) mice were isolated and counted. All mice were 5–8 weeks old. Bold numbering indicates a statistically significant difference (Student's *t*-test; *P* < 0.001) between *sek1*^{-/-} and *sek1*^{+/+} or 129/J mice.

probe), neomycin or β-actin. The amount of Sek1 protein was determined by immunoblotting using a rabbit anti-Sek1 antibody reactive against the kinase domain of Sek1 (Upstate Biotechnology).

SAPK, MAPK and p38 kinase assays. ES cells (1 × 10⁶) were treated with anisomycin (10 μg ml⁻¹ for 30 min), sorbitol (0.4 M, 30 min), UV irradiation (5 and 15 min, using DNA Stratalinker (Stratagene)), or heat shock (43 °C, 30 min). Thymocytes (5 × 10⁶) were activated with PMA (50-ng ml⁻¹) and the Ca²⁺ ionophore A23187 (500 ng ml⁻¹) for 5, 10, 15 and 30 min as described^{2,19}. Cells were lysed in 0.5% NP-40 lysis buffer and MAPKs/ERKs, p38 and SAPK/JNKs were immunoprecipitated for 1 h at 4 °C using a mixture of polyclonal rabbit anti-Erk1/anti-Erk2 IgG (Santa Cruz), polyclonal rabbit anti-p38 (gift from B. Zanke), or polyclonal rabbit anti-SAPK/JNK IgG reactive against all SAPK/JNK isoforms². SAPK/JNK, p38 and MAPK were assayed using a glutathione-S-transferase (GST)-c-Jun fusion protein or MBP as *in vitro* substrates^{2,7}. Erk1/2, SAPK/JNKs and p38 were quantified by immunoblotting.

Immunofluorescence. Single-cell suspensions of thymocytes and mesenteric lymph node cells from *sek1*^{-/-} chimaeric, *sek1*^{+/+} chimaeric and 129/J control mice were stained with anti-CD4 (PE-labelled), anti-CD8 (FITC), anti-CD3ε (PE), anti-TCRαβ (FITC), anti-CD69 (FITC), anti-HSA (FITC), anti-CD44 (FITC), anti-CD5 (FITC) or anti-H-2K^b (FITC). All antibodies were from PharMingen. Samples were analysed using a FACScan (Becton Dickinson).

RAG complementation. For generation of chimaeric mice, two different *sek1*^{-/-} ES cell clones (nos 1–6 and 1–21) and a parental *sek1*^{+/+} E14K ES cell clone were injected into 3.5-day *rag2*^{-/-} blastocysts and transferred to pseudopregnant foster mothers. Mice were maintained under pathogen-free conditions in accordance with the guidelines of the Canadian Medical Research Council. ES cell culture, G418 selection, blastocyst injection and transfer were all done as before¹³. To confirm that T cells were homozygous for the *sek1* mutation, CD4⁺CD8⁺ thymocytes were cell-sorted and analysed using PCR. All CD4⁺CD8⁺ thymocytes from *sek1*^{-/-} chimaeric mice were homozygous for the *sek1* mutation (not shown). Data were all obtained from two independently derived *sek1*^{-/-} ES-cell clones (1–6 and 1–21), with comparable results.

Induction of apoptosis. Freshly isolated thymocytes (1 × 10⁶) from *sek1*^{-/-} and *sek1*^{+/+} mice were plated in triplicate and activated with anti-CD95, anti-CD3ε, anti-CD28, PMA/Ca²⁺ ionophore or heat shock, as indicated. Cells were collected after 24 h, triple-stained with anti-CD4-PE, anti-CD8-FITC and the chromogen 7-AAD to detect viable cells. The percentage of viable thymocytes was determined by cytometry²⁰. For induction of apoptosis in ES cells, confluent *sek1*^{+/+}, *sek1*^{+/+} and *sek1*^{-/-} ES cells were treated with anisomycin (10 μg ml⁻¹, 30 min). The doubling times of *sek1*^{-/-} ES cell lines were similar to those of parental *sek1*^{+/+} and *sek1*^{+/+} ES cells. Differences between *sek1*^{-/-} and wild-type *sek1*^{+/+} double-positive thymocytes were statistically significant (*P* < 0.001; Student's *t*-test) when cell death was induced by anti-CD95 (0.1 and 1 μg ml⁻¹) or anti-CD3ε (2 and 10 μg ml⁻¹).

Received 14 November; accepted 12 December 1996.

- Derijard, B. *et al.* *Cell* **76**, 1025–1037 (1994).
- Kyriakis, J. M. *et al.* *Nature* **369**, 156–160 (1994).
- Minden, A. *et al.* *Science* **266**, 1719–1723 (1994).
- Pombo, C. M. *et al.* *J. Biol. Chem.* **269**, 26546–26551 (1994).
- Westwick, J. K., Bielawska, A. E., Dbaido, G., Hannun, Y. A. & Brenner, D. A. *J. Biol. Chem.* **270**, 22689–22692 (1995).
- Verheij, M. *et al.* *Nature* **380**, 75–79 (1996).
- Sanchez, I. *et al.* *Nature* **372**, 794–798 (1994).
- Derijard, B. *et al.* *Science* **267**, 682–685 (1995).
- Lin, A. *et al.* *Science* **268**, 286–290 (1995).
- Han, J., Lee, J. D., Bibbs, L. & Ulevitch, R. J. *Science* **265**, 808–811 (1994).

- Moriguchi, T., Kawasaki, H., Matsuda, S., Gotoh, Y. & Nishida, E. *J. Biol. Chem.* **270**, 12969–12972 (1995).
- Meier, R., Rouse, J., Cuenda, A., Nebreda, A. R. & Cohen, P. *Eur. J. Biochem.* **236**, 796–805 (1996).
- Fischer, K. D. *et al.* *Nature* **374**, 474–477 (1995).
- Zanke, B. W. *et al.* *Curr. Biol.* **6**, 606–613 (1996).
- Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J. & Greenberg, M. E. *Science* **270**, 1326–1331 (1995).
- Kuida, K. *et al.* *Science* **267**, 2000–2003 (1995).
- Smith, C. A., Williams, M., Kingston, R., Jenkinson, E. H. & Owen, J. J. T. *Nature* **337**, 181–184 (1989).
- Kruisbeek, A. M. & Amsen, D. *Curr. Opin. Immunol.* **8**, 233–244 (1996).
- Su, B. *et al.* *Cell* **77**, 727–736 (1994).
- Schmid, I., Uittenbogaart, C. H. & Giorgi, J. V. *Cytometry* **15**, 12–20 (1994).

Acknowledgements: We thank J. Allison, S. Nagata and B. Zanke for reagents; H. Takimoto and R. Amakawa for technical support; and H. W. Mittrücker, I. Kozieradzki, A. Hakem, A. Wakeham, M. Bachmann, D. Bouchard, S. Gardner, C. Sirard, M. Saunders, S. Nishina, L. Zhang and S. Bagby for comments. J.R.W. and E.R. are supported by grants from the MRC of Canada; K.D.F. and A.B. are supported by the MRC and National Cancer Institute of Canada.

Correspondence and requests for materials should be addressed to J.P. (e-mail: jpenning@amgen.com).

Bcl-x_L forms an ion channel in synthetic lipid membranes

Andy J. Minn[†], Patricio Vélez[‡], Sharon L. Schendel[§], Heng Liang[†], Steven W. Muchmore[†], Stephen W. Fesik^{||}, Michael Fill[‡] & Craig B. Thompson^{*†#}

* Gwen Knapp Center for Lupus and Immunology Research, † Committee on Immunology, and # Howard Hughes Medical Institute, Department of Medicine, and Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, Illinois 60637, USA

‡ Department of Physiology, Cardiovascular Institute, Loyola University of Chicago Stritch School of Medicine, Maywood, Illinois 60153, USA

§ Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907, USA

|| NMR Research and ¶ Protein Crystallography, Pharmaceutical Discovery Division, Abbott Laboratories, Abbott Park, Illinois 60064, USA

Bcl-2-related proteins are critical regulators of cell survival that are localized to the outer mitochondrial, outer nuclear and endoplasmic reticulum membranes^{1–4}. Despite their physiological importance, the biochemical function of Bcl-2-related proteins has remained elusive. The three-dimensional structure of Bcl-x_L, an inhibitor of apoptosis, was recently shown to be similar to the structures of the pore-forming domains of bacterial toxins⁵. A key feature of these pore-forming domains is the ability to form ion channels in biological membranes^{6,7}. Here we demonstrate that Bcl-x_L shares this functional feature. Like the bacterial toxins, Bcl-x_L can insert into either synthetic lipid vesicles or planar lipid bilayers and form an ion-conducting channel. This channel is pH-sensitive and becomes cation-selective at physiological pH. The ion-conducting channel(s) formed by Bcl-x_L display multiple conductance states that have identical ion selectivity. Together, these data suggest that Bcl-x_L may maintain cell survival by regulating the permeability of the intracellular membranes to which it is distributed.

Bcl-2-related proteins play an important role in regulating cell survival in response to a variety of apoptotic stimuli including genotoxic damage, growth factor withdrawal and signal transduction through death-inducing receptors such as Fas^{1,2}. However, the intracellular localization of Bcl-2-related proteins does not suggest a primary role in either intranuclear events or plasma membrane-associated signal transduction. Bcl-2-related proteins are selectively targeted to their intracellular sites by a carboxy-terminal hydrophobic domain^{8,9}. The three-dimensional structure of Bcl-x_L in the absence of this membrane-anchoring domain consists of two central hydrophobic helices surrounded by five amphipathic helices⁵. This structure bears noticeable similarity to the pore-forming domains of several bacterial toxins including colicins A and E1, and diphtheria toxin^{10,11}. Among the bacterial toxins, the encasement of two hydrophobic helices by surrounding amphi-

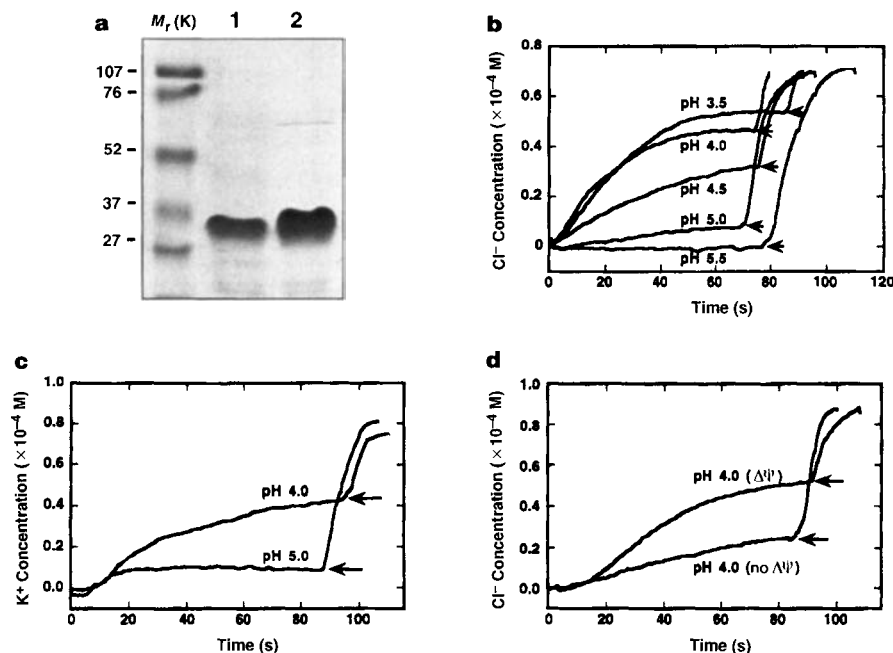


Figure 1 Bcl- x_L forms pores in synthetic lipid vesicles. **a**, An analytical gel of purified recombinant Bcl- x_L preparations used in this study. Recombinant human Bcl- x_L prepared as described in Methods was resolved on a 12.5% SDS-PAGE gel and stained with Coomassie brilliant blue. The molecular masses of the markers (M_r) are indicated to the left of the gel. Lane 1 and lane 2 are two of the four independent protein preparations used in this study, all of which gave comparable results. **b**, A graph of Bcl- x_L -induced Cl^- efflux or **c**, K^+ efflux from synthetic lipid vesicles. KCl-loaded, negatively charged, large unilamellar vesicles were

added to 10 mM dimethylglutarate buffer at the indicated pH values, to give a 200-fold inside:outside KCl gradient. An inside-negative Nernst diffusion potential was induced by the addition of valinomycin. Bcl- x_L was added (time = 0) and ion efflux was measured using a Cl^- -sensitive electrode or a K^+ -sensitive electrode. Triton X-100 was added (arrow) to determine the total encapsulated Cl^- or K^+ . **d**, A graph comparing Cl^- efflux in the presence ($\Delta\psi$) and absence (no $\Delta\psi$) of an inside-negative transmembrane potential induced by valinomycin.

pathic helices allows the proteins to be water soluble yet remain competent to insert into biological membranes after undergoing a conformational change^{6,7}.

To determine if the structural similarity of Bcl- x_L to bacterial toxins extends to functional similarity, the pore-forming ability of Bcl- x_L was tested using synthetic lipid vesicles in an assay that has been used extensively to study the pore-forming properties of bacterial toxins^{6,7}. Assays of ion efflux from solute-loaded vesicles allow macroscopic characterization of channel formation and require membrane insertion of a large number of molecules in order to generate measurable ion efflux¹². For the bacterial colicins and diphtheria toxin, the conformational change that precedes membrane insertion and pore formation is facilitated by combinations of transmembrane potential, acidic pH, and/or negatively charged lipids^{6,7}. Purified recombinant Bcl- x_L lacking the C-terminal hydrophobic domain was added to KCl-loaded lipid vesicles that had an inside-negative transmembrane potential (Fig. 1a). At solution pH values at or above 5.5, Bcl- x_L failed to induce significant Cl^- efflux (Fig. 1b). In contrast, as the solution pH was lowered to values between 5.5 and 3.5, there was a progressive increase in Bcl- x_L -mediated Cl^- efflux. Similar results were obtained when K^+ efflux was measured (Fig. 1c).

The ability of Bcl- x_L to induce membrane permeability was dependent on the presence of negatively charged lipids in the vesicles (10% or greater). No ion efflux was observed from vesicles composed solely of neutral lipids, regardless of pH (data not shown). The requirement for negatively charged lipids suggests that the ability of Bcl- x_L to permeabilize membranes is facilitated by electrostatic interactions between the protein and the lipid surface. However, the ability of Bcl- x_L to cause membrane permeability was specific, because no ion efflux was induced by equivalent amounts of carbonic anhydrase or lysozyme (data not shown), proteins that electrostatically bind to but do not insert through negatively

charged membrane surfaces¹³. Because Bcl- x_L has a net negative surface charge near neutral pH ($pI = 4.8$), low pH may promote the aqueous-phase to lipid-phase transition by causing charge neutralization that results in more favourable electrostatic or hydrophobic interactions with a negatively charged membrane. The presence of a transmembrane potential facilitated Cl^- efflux from Bcl- x_L -permeabilized membranes but was not essential (Fig. 1d). Although the recombinant Bcl- x_L used in this study lacks the hydrophobic C-terminal membrane anchoring domain that concentrates the protein to biological membranes, a dose-titration at pH 4.0 revealed reproducible ion efflux using concentrations of Bcl- x_L as low as 500 ng ml⁻¹ (data not shown).

In order to confirm the ability of Bcl- x_L to induce membrane permeability and to define the nature of this permeability, we examined the incorporation of Bcl- x_L into planar lipid bilayers. At pH 4.0, Bcl- x_L incorporated into planar lipid bilayers and gave rise to current fluctuations that rapidly accumulated over time (Fig. 2a). Step-like current fluctuations representing channel openings to discrete conductance states could be observed shortly after the onset of current activity (Fig. 2b). However, as Bcl- x_L -induced membrane conductance increased, it became difficult to resolve individual step-like channel activity, presumably because of the accumulation of multiple channel-forming complexes (Fig. 2b, c). Nevertheless, under such conditions a mean channel conductance could be estimated by summing single-channel sweeps at various membrane potentials. The current/voltage (I/V) plot generated demonstrates a simple ohmic dependence between voltage and current (Fig. 2c, d). In the presence of a 150:15 mM asymmetric KCl gradient, which allows for the determination of ion selectivity, the direction of the current fluctuations reversed at 0 mV, suggesting that the permeability pathway formed by Bcl- x_L does not discriminate between K^+ and Cl^- at pH 4.0 ($P_K/P_{\text{Cl}} = 1.05$, where P is permeability).

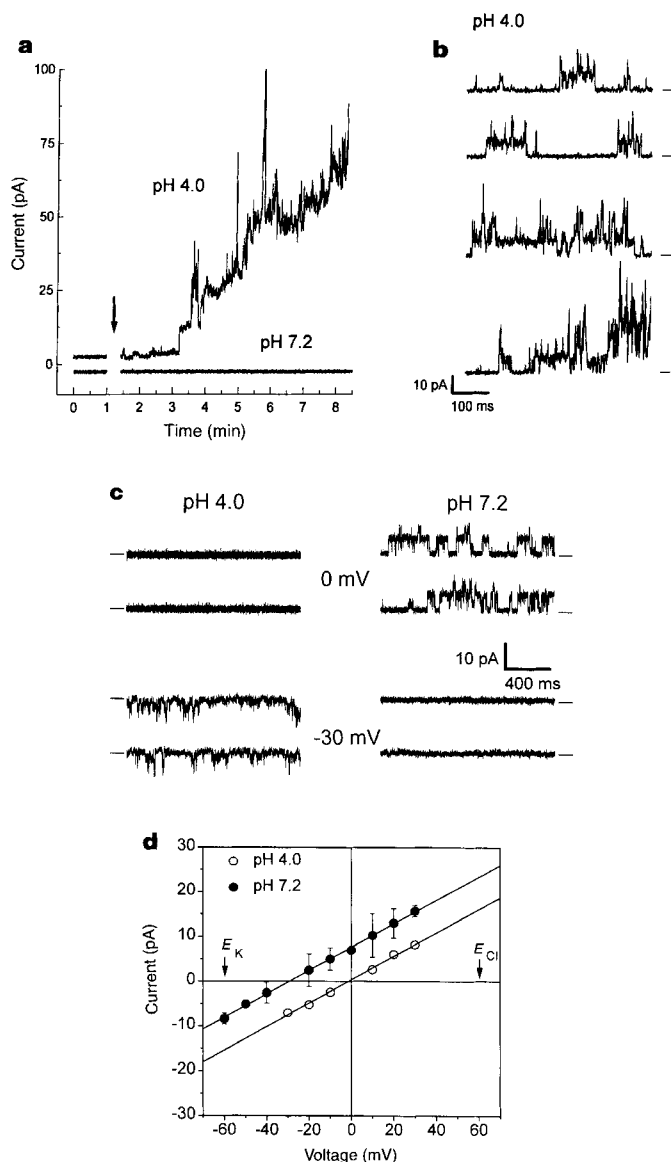


Figure 2 The properties of the Bcl-x_L channel are pH-sensitive. **a**, Bcl-x_L-induced membrane conductance of planar lipid bilayers is enhanced by low pH. Bcl-x_L was added to one side (*cis*) of a planar lipid bilayer in the presence of a 150:50 mM KCl (*cis:trans*) asymmetric gradient and membrane conductance was measured. The pH of both sides was either buffered at pH 4.0 with 50 mM NaC₂H₃O₂ or at pH 7.2 with 10 mM HEPES. The holding potential was 20 mV. The arrow indicates the time at which the protein was added. At pH 7.2, current fluctuations were not observed until 27 ± 17 min (mean $\pm$ s.d.). Both current tracings were offset by ± 2 pA for clarity. **b**, Sample channel recordings at pH 4.0. The zero current levels are marked with a dash. The holding potential was 30 mV. **c**, Sample channel recordings at pH 4.0 and pH 7.2. The holding potentials are indicated. **d**, Current-voltage plots summarizing data collected at pH 4.0 (open circles, $n = 4$) and at pH 7.2 (filled circles, $n = 5$). At pH 4.0, mean current was estimated from ensemble currents generated by summing 16 single-channel sweeps at each potential. At pH 7.2, the predominant individual current unit was measured at each potential. The conductances shown by the I/V plot is about 276 pS at both pH 4.0 and pH 7.2. The theoretical equilibrium potentials for K⁺ and Cl⁻ are indicated.

Physiologically, it is unlikely that Bcl-x_L encounters the extreme acidic pH at which we first observed pore-forming activity. Therefore, we examined pH values closer to the physiological range. Unlike the results obtained at pH 4.0 where almost immediate current fluctuation was observed after addition of Bcl-x_L, at pH 7.2 the average time to the onset of current fluctuations increased to

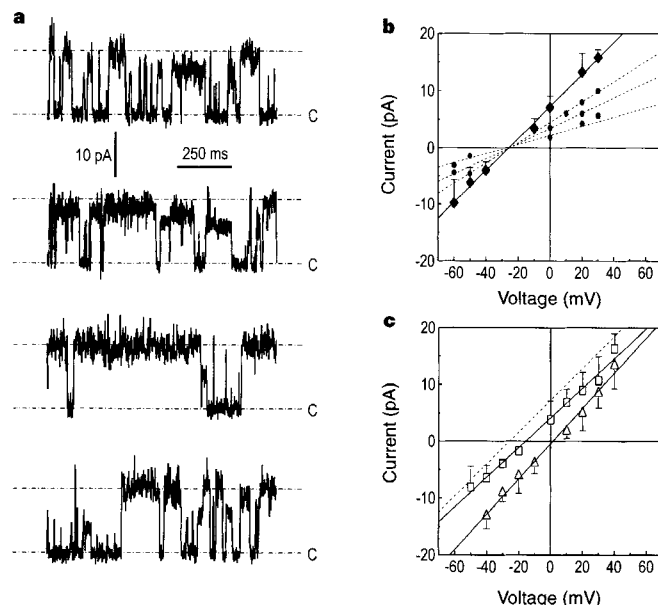


Figure 3 Bcl-x_L forms multi-conductance channels selective for monovalent cations. **a**, Sample channel recordings. Bcl-x_L was added to one side (*cis*) of a planar lipid bilayer in the presence of a 150:15 mM KCl (*cis:trans*) asymmetric gradient at pH 7.2. The closed state (**c**), that is, zero current level, and the predominant open state are marked with dashed lines. **b**, Current-voltage plot of multiple Bcl-x_L conductance states. The predominant conductance state (large diamonds) is 276 ± 28 pS ($n = 11$). Smaller conductance states were also resolved (small circles) and have conductances of 179 ± 11 pS, 134 ± 13 pS and 80 ± 9 pS ($n = 3$). **c**, Current-voltage plot demonstrating cation selectivity profile. Dashed line represents the predominant conductance state (276 pS) when Bcl-x_L is added to 150:15 mM KCl (*cis:trans*) gradient. With the addition of 15:150 mM NaCl (triangles) to the KCl gradient, the reversal potential shifts to +1 mV and the predominant conductance is 301 pS ($n = 3$). With the addition of 0.1:10 mM CaCl₂ (squares) to the KCl gradient the reversal potential shifts to -18 mV and the predominant conductance is 261 pS ($n = 3$).

27 ± 17 min (mean $\pm$ s.d.) (Fig. 2a and data not shown). As at pH 4.0, step-like current fluctuations representing channel activity were observed. Furthermore, because the accumulation of channel activity was much slower at pH 7.2, individual current units could be examined over extended periods of time (Fig. 2c). An I/V plot generated from measuring the major individual current units observed at pH 7.2 also demonstrates a simple ohmic relationship between voltage and current (Fig. 2d). However, in contrast to observations made at pH 4.0, the step-like current events at pH 7.2 had a reversal potential of -30 mV in the presence of a 150:15 mM asymmetric KCl gradient. The shift in the reversal potential toward the theoretical K⁺ equilibrium potential ($E_K = -60$ mV) indicates that the Bcl-x_L channel is cation-selective at pH 7.2 ($P_K/P_{Cl} = 4.31$). Similar results were observed at pH 6.0 (data not shown).

At both pH 4.0 and pH 7.2 the Bcl-x_L channel exhibited multiple conductance states as shown by channel openings that resulted in current fluctuations to various discrete levels (Figs 2b and 3a). The predominant conductance observed was 276 ± 28 pS ($n = 11$) as shown by the I/V plots in Figs 2d and 3b. Three other common conductance states of 179 ± 11 pS, 134 ± 13 pS, and 80 ± 9 pS could also be reliably measured (Fig. 3b). Smaller conductance states could not be clearly resolved. At pH 7.2, all conductance states exhibited the same reversal potential of -30 mV, suggesting that they have similar ion selectivity between K⁺ and Cl⁻. As seen in Fig. 3c, after incorporation of Bcl-x_L into lipid bilayers in the presence of a 150:15 mM KCl (*cis:trans*) asymmetric gradient, addition of 15:150 mM NaCl gradient resulted in a shift of the reversal potential

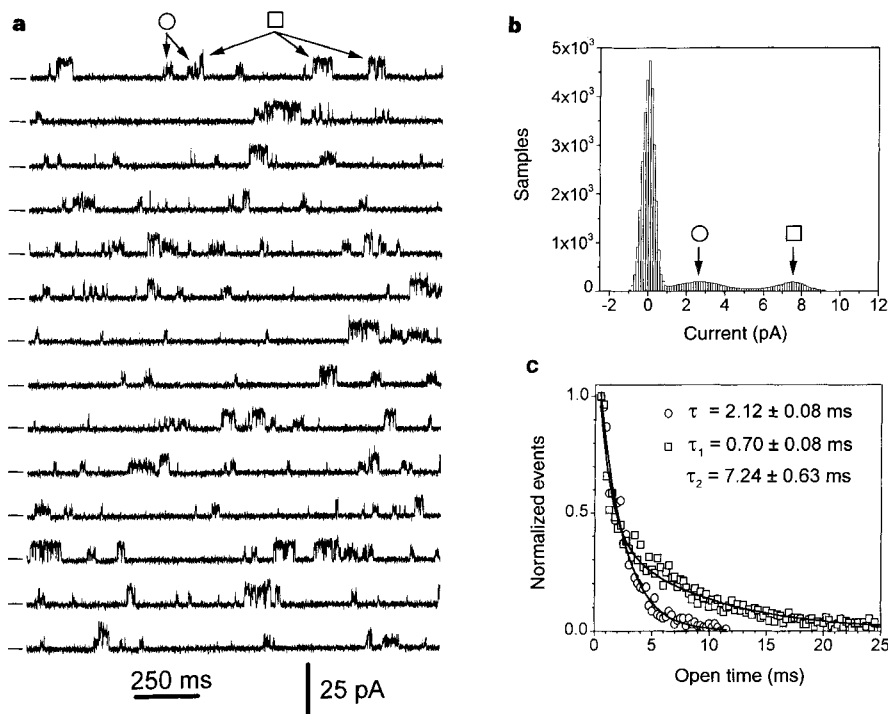


Figure 4 The multi-conductance channels formed by Bcl- x_L have complex opening kinetics. **a**, A 90-s continuous channel recording. Bcl- x_L was added to one side (cis) of a planar lipid bilayer in the presence of a 150:15 mM KCl (cis:trans) asymmetric gradient at pH 7.2. The zero current levels are marked by a dash. A large roughly 270 pS (square) and small roughly 85 pS (circle) conductance state repeatedly occurred over an extended period of time. **b**, All-points amplitude histogram generated from the recording presented in **a**. The large peak repre-

sents the closed current level. The other peaks represent the two open current levels. The line is a gaussian fit to the binned data (bin width = 0.125 pA). **c**, Open time histograms generated from more than 4,000 events to each conductance state. Openings to the large conductance state are best characterized with two time constants (0.70 and 7.24 ms) and openings to the small conductance state are best characterized by a single time constant (2.12 ms). Lines are exponential fits to the binned data (bin width = 0.25 ms) from each data set.

to 0 mV, indicating that the Bcl- x_L channel does not distinguish between K^+ and Na^+ ($P_K/P_{Ca} = 0.966$). Addition of an asymmetric $CaCl_2$ gradient shifted the reversal potential to -18 mV demonstrating that the Bcl- x_L channel can distinguish between K^+ and Ca^{2+} , but only poorly ($P_K/P_{Ca} = 2.15$). Thus, Bcl- x_L forms channels at physiological pH with the following ion selectivity sequence: $K^+ = Na^+ > Ca^{2+} > Cl^-$.

Typically, the multiple conductance states that result from Bcl- x_L incorporation into planar lipid bilayers temporally overlap. However, by holding the membrane potential at or near 0 mV following Bcl- x_L incorporation, stable, individual channel activity could be resolved over an extended period of time (Fig. 4a). Under these conditions, both large and small conductance states repeatedly occurred, providing the opportunity to define the opening kinetics of the Bcl- x_L channel. A current amplitude histogram and a channel open-time histogram were generated from the 90s recording shown in Fig. 4a. Open events were segregated into two groups based on conductance (Fig. 4b). The small conductance opening events of about 85 pS (circles) could be modelled as a single population with a mean open time of 2 ms (Fig. 4c). In contrast, the large conductance opening events of about 270 pS (squares) could not be modelled by a single time constant, suggesting that the large conductance opening events represent a more complex channel. Such a channel could result from the cooperation of several individual pore-forming proteins. Alternatively, an individual pore-forming protein could adopt distinct conformations, resulting in multiple conductance states.

Bcl- x_L has been localized to the outer mitochondrial membrane, the outer nuclear envelope, and the endoplasmic reticulum (ER)². All of these sites have the potential to influence metabolic processes through the regulation of ion permeability. The ER membrane is the

site for the regulation of calcium distribution between the ER lumen and the cytoplasm. Interestingly, Bcl-2 has been reported to influence calcium distribution between these two biological spaces¹⁴, and at least one form of apoptosis has been reported to be inhibited when Bcl-2 was targeted solely to the ER¹⁵. The regulation of ion movement through the outer membranes of the nuclear envelope and the mitochondria has been less appreciated, but the permeability of these membranes is of significant biological importance. For example, the depletion of calcium from the intermembrane space that separates the outer and inner nuclear membrane results in the collapse of nuclear pores and in the inhibition of nuclear transport¹⁶. Electrophysiological studies have revealed that the outer mitochondrial membrane is the site of several ion channels, and evidence suggests that the permeability of these channels is regulated¹⁷. One role for these outer mitochondrial membrane channels may be in controlling mitochondrial permeability transition¹⁸, a process that has been suggested to be critical in apoptosis and has been shown to be inhibited by Bcl-2 (ref 19). Interestingly, outer mitochondrial membrane channels with ion selectivity and conductance properties similar to Bcl- x_L have been reported¹⁷.

The channel properties of Bcl- x_L may be modified by factors not included in the assays used in this study. Like bacterial pore-forming domains, the Bcl- x_L pore-forming domain normally requires additional sequences to target it to biologically relevant membranes (the C-terminal hydrophobic domain). The ability of Bcl- x_L to form a channel may be further modified by the composition and properties of the membranes to which it is attached *in vivo* or by interaction with other proteins that regulate membrane permeability. For example, the interaction of Bcl- x_L with other Bcl-2-related proteins may regulate membrane insertion, ion selectivity and/or conduc-

tance. Although we have demonstrated that Bcl-x_L forms an ion-selective channel, it is also possible that the relevant molecule that Bcl-x_L conducts in order to regulate apoptosis is not an ion. Cytochrome c is a protein that resides in the mitochondrial inter-membrane space and its redistribution to the cytoplasm has been suggested to promote apoptosis through the activation of interleukin-1 β -converting enzyme (ICE)-like proteases^{20,21}. Although the pore-forming domain of diphtheria toxin forms an ion-conductive channel, one important function of this domain is to facilitate passage of another protein, the A fragment, through the endosomal membrane⁶. Thus, it is possible that Bcl-x_L also regulates the passage of proteins.

Using two independent methodologies, we have determined that Bcl-x_L forms an ion channel. This channel is pH-sensitive, cation-selective, and exhibits multiple conductance states with complex opening kinetics. These properties are similar to those reported for the pore-forming domain of one or more of the bacterial toxins that include diphtheria toxin, delta-endotoxin and colicins^{6,7,22}. For example, similar to Bcl-x_L, diphtheria toxin becomes less cation selective at low pH, and low pH also enhances the channel activity of both diphtheria toxin and the colicins^{6,7,23}. Like Bcl-x_L, delta-endotoxin and/or colicin E1 have been reported to have multiple conductance states with complex opening kinetics and comparable ion selectivities^{7,22,24}. However, the collective channel properties of Bcl-x_L appear unique. Although Bcl-x_L shares structural and functional similarities with bacterial pore-forming domains, the ability of Bcl-x_L to promote cell survival may not result solely from its ability to form a channel. For example, although some eye lens crystallins have structural and functional similarities to metabolic or detoxification enzymes with which they are thought to share common ancestors, the shared enzymatic features are not sufficient to account for the functional properties of these crystallins^{25,26}. Nevertheless, this study demonstrates for the first time a biochemical function of a Bcl-2-related protein. The regulation of membrane permeability by Bcl-2-related proteins may be important for their ability to modulate cell survival in response to diverse stimuli. □

Methods

Protein purification. Recombinant human Bcl-x_L protein containing a six amino-acid C-terminal histidine tag was expressed in *Escherichia coli* strain HMS174(DE3) and purified by affinity chromatography on a nickel-IDA column (Invitrogen) as previously described⁵. Further purification was achieved by ion-exchange chromatography on a Q column. The Bcl-x_L used in this study lacks the C-terminal transmembrane region. Additional studies that gave similar results were performed on a Bcl-x_L mutant that also lacks an unstructured region comprising amino acids 45–84, which was previously shown to be dispensable for anti-apoptotic activity⁵.

Synthetic lipid vesicle studies. Large unilamellar vesicles composed of 40% 1,2 dioleoylphosphatidylglycerol and 60% 1,2 dioleoylphosphatidylcholine (Avanti Polar Lipids) were prepared as previously described²⁷. The resulting liposomes were then diluted 200-fold to a concentration of 0.05 mg ml⁻¹ in 10 mM dimethylglutarate, 100 mM choline nitrate²⁷ and 2 mM CaNO₃ titrated to the appropriate pH with NaOH. Valinomycin was added at 15 nM to generate an inside-negative Nernst diffusion potential²⁷. Valinomycin is a K⁺-selective ionophore which can establish a transmembrane potential in the absence of a significant change in the K⁺ concentrations inside or outside of the vesicles²⁸. Bcl-x_L was added at a concentration ranging from 500 ng ml⁻¹ to 10 μ g ml⁻¹. Cl⁻ efflux was measured with a Cl⁻-specific electrode (Orion 94-17B) coupled to a double-junction calomel reference electrode (Orion 90-02). K⁺ efflux was measured with a K⁺-specific electrode (Orion 93-19).

Planar lipid bilayer studies. Planar lipid bilayers were formed across a 200- μ m diameter aperture in the wall of a Delrin cup. Lipid bilayer-forming solution contained 40% phosphatidylserine and 60% phosphatidylcholine (Avanti Polar Lipids) at a concentration of 50 mg ml⁻¹ in *n*-decane. Protein was added to one side of the bilayer, defined as *cis*. The concentration of protein added ranged from 3 μ g ml⁻¹ to 78 μ g ml⁻¹. The other side of the bilayer was defined as *trans* and was the virtual ground. Solutions contained 150:15 mM

KCl (*cis:trans*) and were buffered at either pH 4.0 with 50 mM NaC₂H₃O₂ or pH 7.2 with 10 mM HEPES. Both the *cis* and the *trans* compartments were connected to separate chambers containing a AgCl₂ electrode by a KCl bridge. A custom current/voltage conversion amplifier was used to optimize single-channel recording²⁹. The data were digitized at 2–4 kHz and filtered at 0.75–1.0 kHz using a 12 bit A/D-D/A converter (Axon Instruments) and an 8-pole Bessel filter, except for data demonstrating the time to the onset of current activity, which was digitized at 300 Hz and filtered at 5 Hz. Data were acquired and analysed using pClamp software (Axon Instruments). Curve fits were done using the Marquardt least-squares algorithm.

Received 31 October; accepted 20 December 1996.

- White, E. *Genes Dev.* **10**, 1–15 (1996).
- Yang, E. & Korsmeyer, S. J. *Blood* **88**, 386–401 (1996).
- Lithgow, T., van Driel, R., Bertram, J. F. & Strasser, A. *Cell Growth Differ.* **5**, 411–417 (1994).
- Krajewski, S. *et al. Cancer Res.* **53**, 4701–4714 (1993).
- Muchmore, S. W. *et al. Nature* **381**, 335–341 (1996).
- London, E. *Biochim. Biophys. Acta* **1113**, 25–51 (1992).
- Cramer, W. A. *et al. Annu. Rev. Biophys. Biomol. Struct.* **24**, 611–641 (1995).
- Chen-Levy, Z. & Cleary, M. L. *J. Biol. Chem.* **265**, 4929–4933 (1990).
- Nguyen, M., Millar, D. G., Yong, V. W., Korsmeyer, S. J. & Shore, G. C. *J. Biol. Chem.* **268**, 25265–25266 (1993).
- Parker, M. W. & Pattus, F. *Trends Biochem. Sci.* **18**, 391–395 (1993).
- Choe, S. *et al. Nature* **357**, 216–222 (1992).
- Davidson, V. L., Heymann, J. B., Zhang, Y. L. & Cohen, F. S. *J. Membr. Biol.* **79**, 105–118 (1984).
- Zakharov, S. D., Heymann, J. B., Zhang, Y. L. & Cramer, W. A. *Biophys. J.* **70**, 2774–2783 (1996).
- Lam, M. *et al. Proc. Natl Acad. Sci. USA* **91**, 6569–6573 (1994).
- Zhu, W. *et al. EMBO J.* **15**, 4130–4141 (1996).
- Perez-Terzic, C., Pyle, J., Jaconi, M., Stehno-Bittel, L. & Clapham, D. E. *Science* **273**, 1875–1877 (1996).
- Mannella, C. A. *Trends Biochem. Sci.* **17**, 315–320 (1992).
- Zoratti, M. & Szabo, I. *Biochim. Biophys. Acta* **1241**, 139–176 (1995).
- Zamzami, N. *et al. J. Exp. Med.* **183**, 1533–1544 (1996).
- Liu, X., Kim, C. N., Yang, J., Jemmerson, R. & Wang, X. *Cell* **86**, 147–157 (1996).
- Martin, S. J. & Green, D. R. *Cell* **82**, 349–352 (1995).
- Slatin, S. L., Abrams, C. K. & English, L. *Biochem. Biophys. Res. Commun.* **169**, 765–772 (1990).
- Hoch, D. H. *et al. Proc. Natl Acad. Sci. USA* **82**, 1692–1696 (1985).
- Raymond, L., Slatin, S. L. & Finkelstein, A. J. *Membr. Biol.* **84**, 173–181 (1985).
- Wistow, G. *Trends Biochem. Sci.* **18**, 301–306 (1993).
- Tomarev, S. I. & Piatigorsky, J. *Eur. J. Biochem.* **235**, 449–465 (1996).
- Peterson, A. A. & Cramer, W. A. *J. Membr. Biol.* **99**, 197–204 (1987).
- Cramer, W. A. & Knaff, D. B. *Energy Transduction in Biological Membranes, a Textbook of Bioenergetics* (Springer, New York, 1990).
- Györke, S. & Fill, M. *Science* **260**, 807–809 (1993).

Acknowledgements: A.J.M. and P.V. contributed equally to this work. We thank R. Miller, C. Rudin, C. Duckett, B. Chang, M. Vander Heiden and T. Lindsten for discussions and review of the manuscript. We also thank M. Cortez for technical assistance, and T. Conway and D. Wang for editorial assistance. This work was supported by grants from the NIH and the Howard Hughes Medical Institute.

Correspondence should be addressed to either M.F. (e-mail: mfill@luc.edu) or C.B.T. (e-mail: cthomps@midway.uchicago.edu).

The C-terminal domain of RNA polymerase II couples mRNA processing to transcription

Susan McCracken, Nova Fong, Krassimir Yankulov, Scott Ballantyne*, Guohua Pan†, Jack Greenblatt†, Scott D. Patterson‡, Marvin Wickens* & David L. Bentley

Amgen Institute, 620 University Avenue, Suite 706, Toronto, Ontario M5G 2C1, Canada

* Department of Biochemistry, University of Wisconsin, 420 Henry Mall, Madison, Wisconsin 53706-1569, USA

† Banting and Best Department of Medical Research and Department of Molecular and Medical Genetics, University of Toronto, Ontario M5G 1L6, Canada

‡ Amgen Inc., Department of Protein Structure, 1840 DeHavilland Drive, Thousand Oaks, California 91320, USA

Messenger RNA is produced by RNA polymerase II (pol II) transcription, followed by processing of the primary transcript. Transcription, splicing and cleavage–polyadenylation can occur independently *in vitro*, but we demonstrate here that these processes are intimately linked *in vivo*. We show that the carboxy-terminal domain (CTD) of the pol II large subunit is

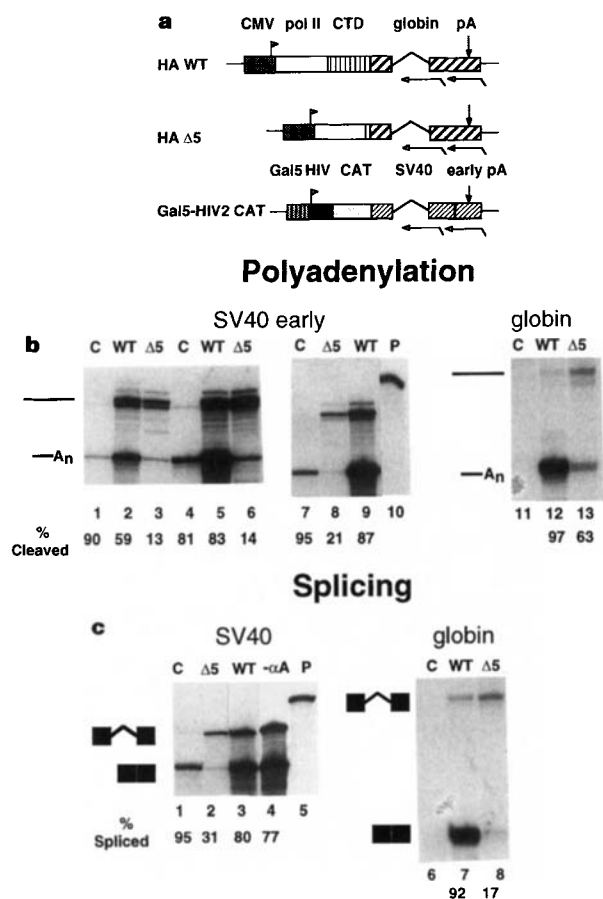


Figure 1 CTD truncation inhibits 3' processing and splicing. **a**, Wild-type (WT) and CTD-truncated ($\Delta 5$) pol II expression plasmids and Gal5-HIV2 CAT reporter. **b**, RNAse protection of cleavage at the SV40 early (lanes 1–9) and rabbit β -globin (lanes 11–13) poly(A) sites. Transcription was activated by Gal4-Sp1 (lanes 1–3) or Gal4-VP16 (lanes 4–9). In lanes 7–9, Gal5-HIV2 CAT was transfected after addition of α -amanitin (see Methods). C, CMV *neo* control; P, probe. **c**, RNAse protection of splicing at the SV40-t intron (lanes 1–4) and rabbit β -globin intron 2 (lanes 6–8). Lanes 1–3 are the same RNAs as in **b**, lanes 7–9. Lanes 1, 2 were exposed for twice as long as lane 3. Lane 4, control without pol II plasmid or α -amanitin.

required for efficient RNA processing. Splicing, processing of the 3' end and termination of transcription downstream of the poly(A) site, are all inhibited by truncation of the CTD. We found that the cleavage-polyadenylation factors CPSF and CstF specifically bound to CTD affinity columns and copurified with pol II in a high-molecular-mass complex. Our demonstration of an association between the CTD and 3'-processing factors, considered together with reports of a similar interaction with splicing factors^{1,2}, suggests that an mRNA 'factory' exists which carries out coupled transcription, splicing and cleavage-polyadenylation of mRNA precursors.

To study the role of the CTD in mRNA production, we transiently transfected cells with vectors expressing α -amanitin-resistant forms of the murine pol II large subunit which had either 5 or 52 heptad repeats in the CTD (consensus, YSPTSPS). These two forms are referred to as $\Delta 5$ (or Δ CTD) and wild type (WT), respectively (Fig. 1a). After a short period of expression, α -amanitin was added to inhibit the host cell's pol II. Using this strategy, it was shown that *in vivo*, as *in vitro*⁴, CTD truncation does not affect the accuracy of initiation but reduces the response to enhancers³. We initially analysed transcripts of a chloramphenicol acetyltransferase (CAT) reporter gene made by wild-type and Δ CTD pol II and found,

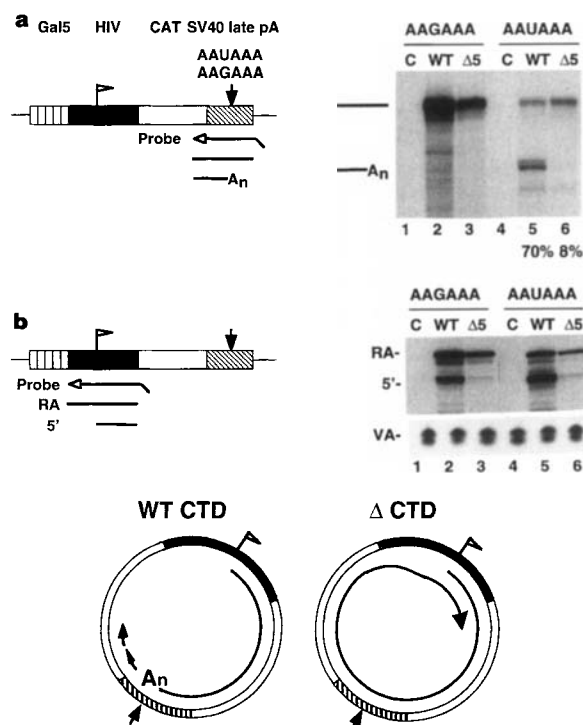


Figure 2 **a**, RNAse protection of SV40 late poly(A) site processing in pre-mRNAs without introns. Transcription of Gal5-HIV2 CAT-AAUAAA and Gal5-HIV2 CAT-AAGAAA was activated by Gal4-VP16. **b**, RNAse protection of the same samples as in **a**, with a 5' probe, shows accumulation of read-around (RA) transcripts relative to 5' ends (5') when AAUAAA is mutated and when the CTD is truncated. Diagram shows the effect of CTD truncation on RA transcription.

surprisingly, that RNA cleavage at the SV40-early poly(A) site was severely inhibited by truncation of the CTD (Fig. 1b; compare $\Delta 5$ lanes 3, 6 and 8 with WT lanes 2, 5 and 9). Inhibition of 3' processing was observed with both strong and weak transcriptional activators (Gal4-Sp1 in lane 3 and Gal4-VP16 in lane 6 of Fig. 1b), showing that it is not an indirect effect of reduced initiation by the Δ CTD polymerase. Furthermore, 3' processing was inhibited even when the CAT reporter was transfected after addition of α -amanitin (Fig. 1b, lanes 7–9). Cleavage at the rabbit β -globin (Fig. 1b, lanes 12, 13) and human α -globin (data not shown) poly(A) sites was also significantly reduced by CTD truncation. We then compared splicing of two introns transcribed by pol II with a wild-type or truncated CTD. Splicing of the SV40-t intron and the rabbit β -globin intron 2 was significantly inhibited by truncating the CTD (Fig. 1c). Splicing was equally efficient in untreated cells and in α -amanitin-treated cells transfected with the wild-type pol II expression vector (Fig. 1c, lanes 3 and 4).

Control experiments were done to eliminate the possibility that inhibition of RNA processing in cells expressing Δ CTD polymerase was due to depletion of unstable processing factors. Inhibition of protein synthesis by addition of cycloheximide plus α -amanitin to cells expressing WT pol II did not affect RNA processing (data not

shown). Furthermore, α -amanitin-treated cells transfected with CMV *neo*, instead of a pol II expression plasmid, produced a small amount of CAT RNA that was correctly spliced and cleaved at the 3' end (Fig. 1b, lanes 1, 4 and 7; Fig. 1c, lane 1), showing that even a greatly reduced level of overall pol II transcription did not inhibit processing. We conclude that inhibition of RNA processing is specific to transcripts made by pol II with a truncated CTD and not those made by full-length pol II.

Inhibition of 3' processing by the Δ CTD polymerase could be an indirect effect of inhibiting splicing of the 3' intron^{5,6}. To test this possibility, we analysed two CAT reporter genes with wild-type or mutant SV40-late poly(A) sites but no introns. 3' processing of the SV40-late poly(A) site was greatly reduced by CTD truncation in the absence of an intron (Fig. 2a, lanes 5 and 6). There was no processing at the AAGAAA mutant site, confirming the sequence specificity of this cleavage reaction (Fig. 2a, lanes 2 and 3). We conclude that inhibition of 3' processing by CTD truncation cannot be accounted for as a secondary effect of reduced splicing.

The AAUAAA and G/U rich elements that are required for 3' processing are also necessary for transcriptional termination, which usually occurs far downstream of the poly(A) site⁷⁻⁹. Mutation of these motifs can cause RNA polymerase II to read around the entire plasmid several times because of failure to terminate⁷. Read-around (RA) transcripts are distinguished in an RNase protection assay because they protect a region of the probe that extends upstream and downstream of the start site (Fig. 2b). Even in the presence of a wild-type AAUAAA motif, read-around transcripts accumulated when the gene was transcribed by the Δ CTD polymerase (Fig. 2b, lane 6, and lower panel). This observation shows that although a wild-type AAUAAA is necessary for pol II termination, it is not sufficient in the absence of the CTD.

To pursue the biochemical basis of the functional link between the CTD and 3' RNA processing, we investigated whether cleavage-polyadenylation factors bind to this domain. HeLa cell nuclear extract was chromatographed sequentially on affinity columns of glutathione S-transferase (GST) and the fusion proteins GST-mutant CTD, GST-wild-type CTD and hyperphosphorylated GST-wild-type CTD (Fig. 3a, lanes 1-4). High-salt eluates of the columns were analysed by western blotting with antibodies against two subunits of CstF and three subunits of CPSF, as well as against poly(A) polymerase (Fig. 3b). CstF and CPSF, but not poly(A) polymerase, bound to hypo- and hyperphosphorylated wild-type CTD but not to GST or mutant CTD. Although similar profiles of total protein eluted from all three CTD columns (Fig. 3a, lanes 8-10), CstF and CPSF were found specifically in the eluates from the two wild-type columns. The fraction of CstF and CPSF that bound the CTD differed between subunits, possibly reflecting unequal amounts of these polypeptides in the HeLa cell extract. CstF and CPSF coimmunoprecipitate (S.M. and D.B., unpublished results) and may bind to the CTD as a complex. Significant binding of TBP¹⁰, perhaps as a component of the TFIID complex, but not of TFIIB, TFIIE or TFIIIF, to the CTD was also detected (Fig. 3b).

CstF binding to the CTD was investigated by translation of its three subunits *in vitro*, followed by binding to GST resins. The p50 subunit bound very efficiently to wild-type CTD but not to GST or mutant CTD (Fig. 3c). The p77 subunit also bound specifically to the CTD but much less efficiently than p50, and p64 did not bind at all. The p50 subunit has seven β -transducin (WD40) repeats at its C terminus (residues 93-431)¹¹. Incomplete translation products (Fig. 3c, top) and a C-terminally deleted form with 2.5 β -transducin repeats (residues 1-211) bound to the CTD weakly compared with full-length p50 (Fig. 3c). CTD binding was severely reduced by N-terminal deletion of residues 1-91 of p50 (Fig. 3c), indicating that the β -transducin repeats are not sufficient for CTD binding.

If 3'-processing factors stably associate with the CTD *in vivo*, they may copurify with pol II. In mammalian cells, pol II exists as high-molecular-mass holoenzyme complexes containing general tran-

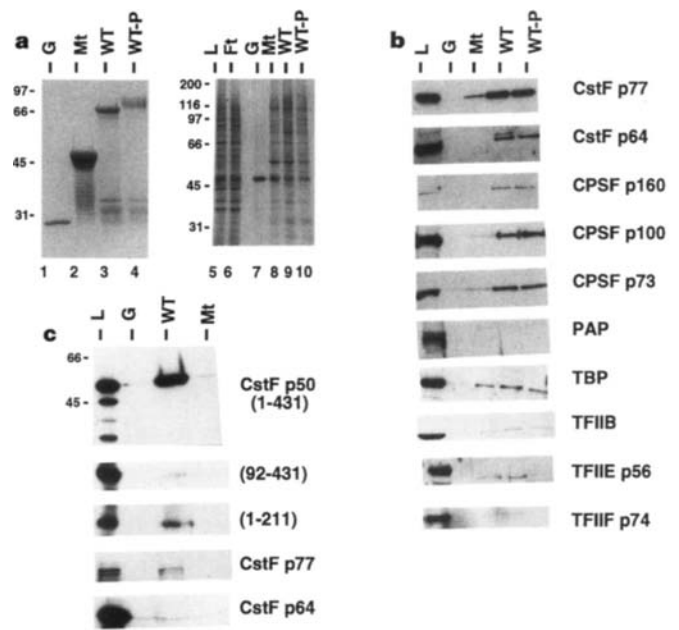


Figure 3 CPSF and CstF bind specifically to the CTD. **a**, Lanes 1-4, Coomassie stain of GST(G), GST-CTD mutant (Mt), GST-CTD wild type (WT) and GST-CTD hyperphosphorylated (WT-P) affinity resins. Lanes 5-10, silver stain of load (L, 0.5 μ l), flow-through (Ft, 0.5 μ l) and 0.6 M NaCl eluates (15 μ l). **b**, Western blots of load (0.083%, 5 μ l) and column eluates (1%, 5 μ l). Note that anti-TFIIE detected a lower- M_r artefact band in addition to p56. **c**, Binding of ³⁵S-Met-labelled *in vitro* translated CstF subunits to the CTD. 10% of the input (L) and 50% of the bound fractions are shown.

scription factors (GTFs)^{12,13}. Holoenzyme was prepared by affinity chromatography on GST-TFIIS, followed by gel filtration; this complex contains TFIID and all the general transcription factors required for initiation (G.P., T. Aso and J.G., manuscript submitted). Western blotting of the eluate from the GST-TFIIS column (Fig. 4, left) showed retention of CPSF (p160) and CstF (p64 and p77) but not of poly(A) polymerase or the coactivator CBP. Gel filtration of the GST-TFIIS eluate (Fig. 4, right) showed that pol II, TFIIIF (rap 74), TFIIF (ERCC3), cyclin C (data not shown) and CstF cofractionated precisely, peaking in fraction 24 with an apparent relative molecular mass (M_r) greater than 2,000K. CPSF copurified extensively with pol II but its peak was in fraction 23, probably because there is some heterogeneity of the complexes.

Our results demonstrate that a full-length RNA pol II CTD is required for efficient splicing and 3' processing *in vivo*. The binding of 3'-processing factors to the CTD provides a plausible molecular explanation for the functional coupling of transcription with cleavage-polyadenylation. CPSF/CstF binding to the CTD probably promotes recruitment to the RNA substrate and thereby enhances the cleavage reaction. This effect can explain why pol I (ref. 14, and D. Zarkower and M.W., unpublished results), pol III (ref. 15) and T7 RNA polymerase (ref. 16, and N.F. and D.B., unpublished results), which lack a CTD, do not produce mRNAs that are efficiently cleaved and polyadenylated.

After completing synthesis of the pre-mRNA, pol II terminates transcription in an AAUAAA-dependent manner. The mechanism that couples termination to polyadenylation is unknown⁷⁻⁹. Our observations that the AAUAAA signal and the CTD are both required for termination (Fig. 2b) and that 3'-processing factors bind to phosphorylated and non-phosphorylated CTD (Fig. 3b) suggest a solution to this problem (Fig. 5). We propose that CPSF and CstF, as well as splicing factors^{1,2}, travel with the hyperphosphorylated elongating polymerase. The CPSF/CstF interaction

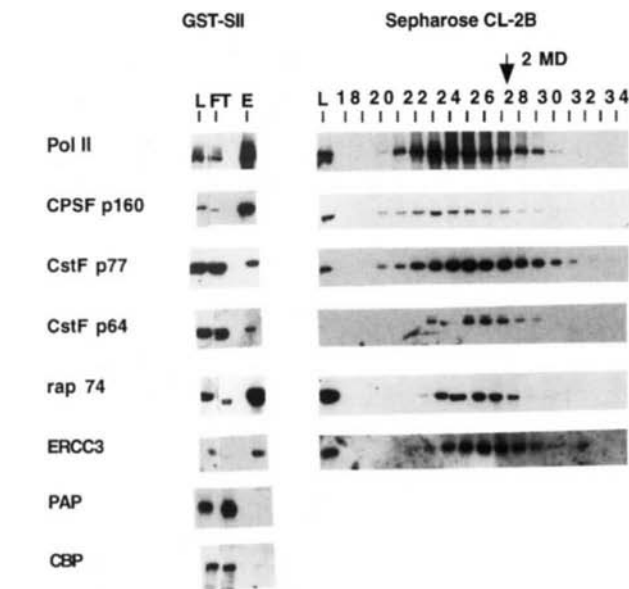


Figure 4 CstF and CPSF copurify with pol II holoenzyme. This complex was purified by affinity chromatography of HeLa extract on GST-TFIIS (SII) and gel filtration on Sepharose CL-2B. Western blots of load (L), flow-through (FT) and 0.325 M NaCl eluate (E) from the GST-TFIIS column (left) and the load (L) and gel filtration column fractions (right). The 2-megadalton Dextran-blue 2000 marker (2 MD; Pharmacia) is indicated. Equal signals for the GST-TFIIS load and eluate fractions signify 1.5% binding.

with the CTD is altered or disrupted when the polymerase transcribes the polyadenylation signal and processing factors bind to the RNA transcript. The altered interaction of the CTD with 3'-processing factors could signal a switch in the polymerase to a termination-competent state. This model is analogous to the modulation of *Escherichia coli* RNA polymerase elongation through binding of phage lambda N protein to the nascent RNA.

The effect of the CTD on splicing (Fig. 1c) may be due to recruitment of splicing factors to the pre-mRNA via protein-protein interactions with the CTD¹⁷. Our results are consistent with observations of co-transcriptional splicing *in vivo*^{18,19} and corroborate recent reports demonstrating an association of SR-family splicing factors with the CTD (refs 1 and 2, and S.M. and D.B., data not shown) and inhibition of *in vitro* splicing by a CTD peptide¹.

Functional^{5,6} and biochemical^{20,21} association between the splicing and 3'-processing machineries has been established. Furthermore, sites of transcription in the nucleus colocalize with sites of splicing^{22,23} and with a fraction of the polyadenylation factor CstF64 (ref. 24). We propose that splicing and polyadenylation are not only coupled to one another, but also to transcription. In particular, we suggest that mature transcripts are produced in an mRNA 'factory' in which the transcriptional, splicing and cleavage-polyadenylation machineries interact through contacts with the pol II CTD (Fig. 5). The copurification of polyadenylation factors with an initiation-competent form of pol II holoenzyme suggests that the 'factory' may be in place at the promoter. This complex is effectively an assembly line that enhances mRNA production by channelling precursors directly from the synthetic machinery to the processing machinery. □

Methods

Transfection and RNA analysis. 293 cells were transfected with 5 µg Gal5-HIV2 CAT reporter, 0.5 µg pSPVA, 5 µg pol II expression vector³ or CMV *neo* and 1 µg Gal4 fusion vector by $\text{Ca}_3(\text{PO}_4)_2$ precipitation. α -amanitin (2.5 µg ml⁻¹) was added after 12–15 h and cells were collected after 65 h.

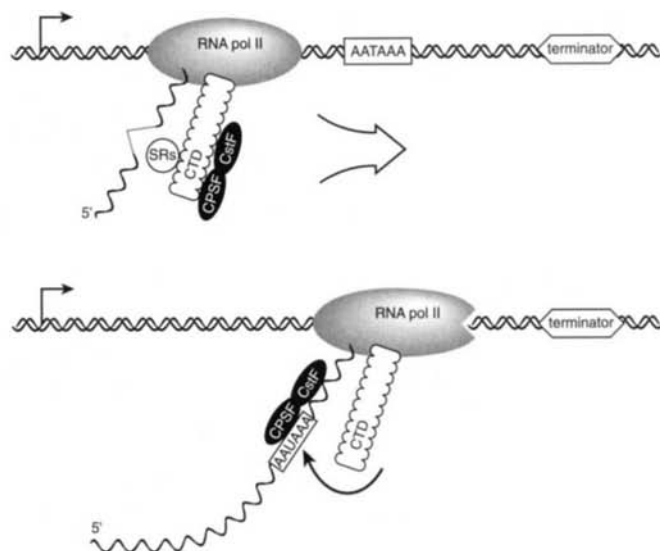


Figure 5 Top, model for an mRNA factory capable of RNA synthesis and processing. Bottom, alteration or reversal of CPSF/CstF binding to the CTD at the poly(A) site switches pol II into a termination-competent state.

Mock-transfected and Δ CTD-expressing cells survive at least three days in α -amanitin. In Fig. 1b, lanes 7–9, and Fig. 1c, lanes 1–3, the Gal5-HIV2 CAT reporter was introduced after 24 h in a second transfection 1 h after addition of α -amanitin. RNA was collected after 72 h for RNase protection²⁵. Antisense riboprobes: SV40 early poly(A) site, *SspI*-linearized pGcat2.2 (ref. 26); SV40 late poly(A) site, PCR fragments of Gal5-HIV2 CAT-AAGAAA/AAUAAA (–205 to +70 relative to AAUAAA); SV40 t-antigen intron 3' splice site, 345-bp *ApoI*–*SspI* fragment; rabbit β -globin poly(A), 220-bp *EcoRI*–*NdeI* fragment; rabbit β -globin intron-2 3' splice site, 180-bp *ApaI*–*BglII* fragment. VA1 RNA was analysed as described²⁵. Gel bands were quantified by Phosphorimager (Molecular Dynamics) and corrected for U content.

Affinity chromatography. GST fusion proteins were immobilized at 4 mg ml⁻¹, except for the mutant CTD, which was at 8 mg ml⁻¹. The mutant CTD has 15 repeats of YSPTAPS²⁷; the wild-type has all 52 repeats of murine pol II (ref. 28). GST-CTD resin was phosphorylated with total HeLa nuclear extract. Antibodies raised against this material specifically recognized pol II. For Fig. 3b, HeLa nuclear extract²⁹ (33 mg in 6 ml buffer D with 0.1 M KCl instead of NaCl) was passed sequentially over four 0.2-ml columns at 4 °C, washed with 2 ml buffer D-0.1 M KCl and eluted with 0.5 ml of buffer D-0.6 M NaCl. 0.3–0.4% of total protein bound to each CTD resin. Ethidium bromide and RNase did not affect CPSF or CstF binding.

³⁵S-Met-labelled CstF p50, p64 and p77 were made by TNT (Promega) from full-length rat cDNAs. The C-terminal deletion (residues 1–211) of p50 was made by linearizing with *DraI* and the N-terminal deletion (92–431) was made by subcloning an *AatII*–*StuI* fragment into pET21d. Translations were diluted 1:10 in 20% glycerol, 100 mM NaCl, 0.05% non-fat dry milk, 0.1% N-P40, 400 µg ml⁻¹ ethidium bromide, 20 mM HEPES, pH 7.9, 0.1 mM EDTA, 2 mM DTT, and bound to GST resins (25 µl) for 1 h at 25 °C, washed 3 times with 0.5 ml binding buffer and eluted in 0.6 M NaCl.

Antibodies. Anti-p64, anti-PAP and anti-p160 were provided by J. Manley, anti-p100 and anti-p73 (ref. 30) by A. Jenny and W. Keller, anti-ERCC3 by J.-M. Egly, and anti-TFIIB by P. O'Hare; anti-CBP (A-22) was from Santa Cruz Biotechnology and anti-TBP was from Upstate Biotechnology. Anti-TFIIE(p56), anti-TFIIF(rap74) and anti-CstF 77 and anti-PAP peptide antisera were raised in rabbits and affinity-purified.

Pol II holoenzyme. Holoenzyme was prepared as described (G.P., T. Aso and

J.G., manuscript submitted). HeLa whole-cell extract (200 mg) in CHB buffer (10 mM HEPES, pH 7.9, 0.2 mM EDTA, 0.2 mM EGTA, 5 mM β -glycerophosphate, 1 mM DTT, 50 μ M ZnCl₂, 0.05% N-P40, 12% glycerol) plus 50 mM NaCl was chromatographed on a 1-ml GST-TFIIIS column (10 mg per ml ligand), washed with 15 ml CHB-50 mM NaCl, then eluted with CHB-325 mM NaCl. 2 ml of eluate was loaded onto a Sepharose CL-2B column (60 $\times$ 1.6 cm; flow rate 0.4 ml min⁻¹) equilibrated with CHB-100 mM NaCl plus 8% glycerol, and 4-ml fractions were collected.

Received 31 July; accepted 22 November 1996.

- Yuryev, A. *et al.* *Proc. Natl. Acad. Sci. USA* **93**, 6975–6980 (1996).
- Mortillaro, M. J. *et al.* *Proc. Natl. Acad. Sci. USA* **93**, 8253–8257 (1996).
- Gerber, H. P. *et al.* *Nature* **374**, 660–662 (1995).
- Zehring, W. A., Lee, J. M., Weeks, J. R., Jokerst, R. S. & Greenleaf, A. L. *Proc. Natl. Acad. Sci. USA* **85**, 3698–3702 (1988).
- Nesic, D., Cheng, J. & Maquat, L. E. *Mol. Cell. Biol.* **13**, 3359–3369 (1993).
- Niwa, M., Rose, S. D. & Berget, S. M. *Genes Dev.* **4**, 1552–1559 (1990).
- Connelly, S. & Manley, J. L. *Genes Dev.* **2**, 440–452 (1988).
- Logan, J., Falck, P. E., Darnell, J. E. J. & Shenk, T. *Proc. Natl. Acad. Sci. USA* **84**, 8306–8310 (1987).
- Whitelaw, E. & Proudfoot, N. *EMBO J.* **5**, 2915–2922 (1986).
- Usheva, A. *et al.* *Cell* **69**, 871–881 (1992).
- Takagaki, Y. & Manley, J. L. *J. Biol. Chem.* **267**, 23471–23474 (1992).
- Ossipow, V., Tassan, J. P., Nigg, E. A. & Schibler, U. *Cell* **83**, 137–146 (1995).
- Maldonado, E. *et al.* *Nature* **381**, 86–89 (1996).
- Smale, S. T. & Tjian, R. *Mol. Cell. Biol.* **5**, 5352–5362 (1985).
- Sisodia, S. S., Sollner, W. B. & Cleveland, D. W. *Mol. Cell. Biol.* **7**, 3602–3612 (1987).
- Mifflin, R. C. & Kellems, R. E. *J. Biol. Chem.* **266**, 19593–19598 (1991).
- Greenleaf, A. L. *Trends Biochem. Sci.* **18**, 117–119 (1993).
- Beyer, A. L. & Osheim, Y. N. *Genes Dev.* **2**, 754–765 (1988).
- Wuarin, J. & Schibler, U. *Mol. Cell. Biol.* **14**, 7219–7225 (1994).
- Lutz, C. S. *et al.* *Genes Dev.* **10**, 325–337 (1996).
- Boelens, W. C. *et al.* *Cell* **72**, 881–892 (1993).
- Zhang, G., Taneja, K. L., Singer, R. H. & Green, M. R. *Nature* **372**, 809–812 (1994).
- Mattaj, J. W. *Nature* **372**, 727–728 (1994).
- Schul, W. *et al.* *EMBO J.* **15**, 2883–2892 (1996).
- Blau, J. *et al.* *Mol. Cell. Biol.* **16**, 2044–2055 (1996).
- Lieber, A., Kiessing, U. & Strauss, M. *Nucleic Acids Res.* **17**, 8485–8493 (1989).
- West, M. & Corden, J. *Genetics* **140**, 1223–1233 (1995).
- Peterson, S. R., Dvir, A., Anderson, C. W. & Dym, W. S. *Genes Dev.* **6**, 426–438 (1992).
- Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. *Nucleic Acids Res.* **11**, 1475–1489 (1983).
- Jenny, A., Minvielle-Bastia, L., Preker, P. & Keller, W. *Science* **274**, 1514–1517 (1996).

Acknowledgements: S.M.C., N.F. and K.Y. contributed equally to this work. We thank J. Blau, P. Atadja and B. McNeil for their contribution; J. Corden, M. West and A. Yuryev for plasmids and for sharing unpublished data; R. Treisman and L. Harrington for discussion; J. Manley, W. Keller, A. Jenny, P. O'Hare, E. Lees, J.-M. Egly, M. Rihaneck, T. Zamborelli, M. Pandes, J. Biron, S. Suggs and the Amgen EST Program for antibodies, HeLa cells and CstF plasmids; and A. Hessel, H. Agah, M. Pandes, P. Courchesne, B. Bolychuk and S. Pang for computer, technical, photographic and secretarial assistance.

Correspondence and requests for materials to D.B. (e-mail: david.bentley@amgen.com).

Structure of the conserved GTPase domain of the signal recognition particle

Douglas M. Freymann, Robert J. Keenan, Robert M. Stroud & Peter Walter

Department of Biochemistry and Biophysics, School of Medicine, University of California, San Francisco, California 94143-0448, USA

The signal-recognition particle (SRP) and its receptor (SR) function in the co-translational targeting of nascent protein-ribosome complexes to the membrane translocation apparatus¹. The SRP protein subunit (termed Ffh in bacteria) that recognizes the signal sequence of nascent polypeptides is a GTPase, as is the SR- α subunit (termed FtsY)^{2,3}. Ffh and FtsY interact directly, each stimulating the GTP hydrolysis activity of the other⁴. The sequence of Ffh suggests three domains: an amino-terminal N domain of unknown function, a central GTPase G domain, and a methionine-rich M domain that binds both SRP RNA and signal peptides^{5,6}. Sequence conservation suggests that structurally similar N and G domains are present in FtsY^{7,8}. Here we report the structure of the nucleotide-free form of the NG fragment of Ffh. Consistent with a role for apo Ffh in protein targeting, the side chains of the empty active-site pocket form a tight network of

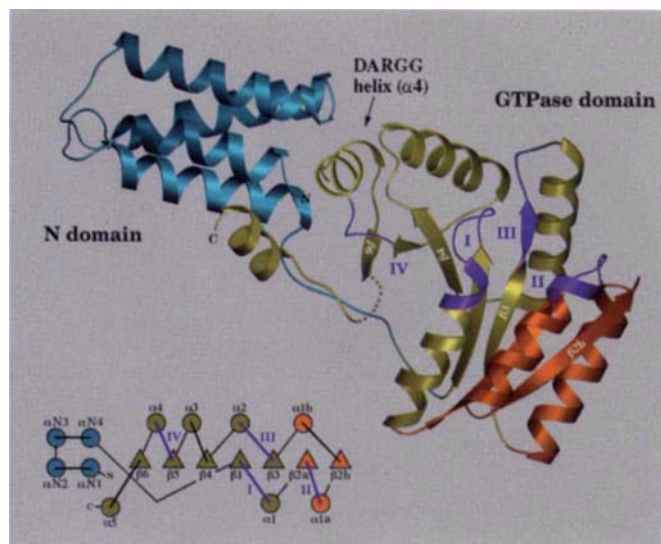


Figure 1 Structure and topology of the NG domain. The four-helix bundle of the N domain (blue) is closely associated with the GTPase domain (green and orange). The loops containing the conserved GTPase sequence motifs I (105–112), II (135–141), III (187–192), and IV (245–248) are highlighted (purple); these define the position of the GTP-binding site. The interface between the two domains occurs primarily along a single, distorted helix (the 'DARGG' helix). The main structural difference distinguishing the GTPase of Ffh from other GTPases is the subdomain, coloured orange, inserted between helix α 1 and strand β 3 of the core GTPase fold. The α -helices and β -strands are numbered in accordance with their structural counterparts in p21^{ras}. An eight-residue loop cannot be seen in the electron-density map and is probably disordered because of the absence of bound nucleotide; it is indicated by the dotted line.

interactions which may stabilize the nucleotide-free protein. The structural relationship between the two domains suggests that the N domain senses or controls the nucleotide occupancy of the GTPase domain. A structural subdomain unique to these evolutionarily conserved GTPases constitutes them as a distinct subfamily in the GTPase superfamily⁹.

The structure of the apo form of Ffh NG (Fig. 1) was determined to a resolution of 2.05 Å by X-ray diffraction using multiple isomorphous replacement (Table 1). The G domain has a β/α fold that is structurally similar to other GTPases in the superfamily including p21^{ras} (Ras)¹⁰, EF-G^{11,12} and transducin G α ¹³. The N domain is a bundle of four antiparallel α -helices, which open at one end, allowing sidechains of the G domain to complete the packing of its hydrophobic core. The protein is thus a single structural unit in which the two sequence domains, N and G, are distinct, yet closely associated. A ten-residue peptide that links the two domains is packed tightly against the protein surface, rendering the NG fragment stable to proteolysis^{5,14}.

The core GTPase fold is a five-stranded β -sheet surrounded by α -helices; loops between the β -strands of the sheet and the α -helices contain four highly conserved sequence motifs (I–IV) which mediate interaction with the bound ribonucleotide¹⁵ (Fig. 1, purple). Two striking structural differences distinguish the G domain of Ffh from other subfamilies of GTPases. The first is the subdomain (Fig. 1, orange), which includes motif II (G-2)¹⁵; this region is highly conserved within, but not between, subfamilies of GTPases, and in Ras and other GTPases it is known to interact with GTPase-activating proteins⁹. As Ffh and FtsY mutually stimulate GTP hydrolysis⁴, the subdomain is likely to function in the interaction between the Ffh and its receptor. It is an insertion of 50 amino acids that extends the core β -sheet by two strands and two helices to form a seven-stranded all-parallel β -sheet. The first

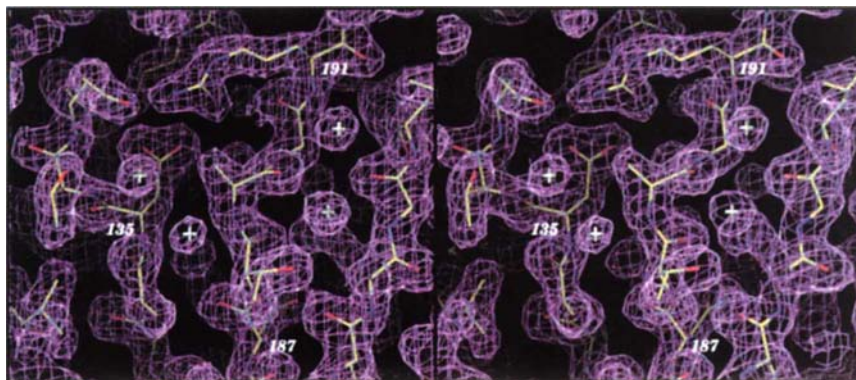


Figure 2 Stereo view of the 2.05-Å resolution electron-density map contoured at 1.25 σ obtained after phase combination with the experimental MIR phases. The positions of several water molecules are marked (+). The side chains of Asp 135

and Arg 191 form a salt bridge. These residues are part of conserved GTPase motifs II and III, respectively, and Arg 191 is proposed to interact with the bound GTP; if so, the side chains must rearrange during the GTPase cycle of Ffh.

connecting loop of the subdomain, between β 2a and α 1a (Fig. 1, insert), contains the conserved motif II, which forms part of the predicted nucleotide-binding pocket. The second striking difference between the G domain and other GTPases occurs at the C-terminal helix. In all other GTPases for which a structure is known, a C-terminal helix packs against the first helix (α 1) of the fold. In the G domain of NG, there is no corresponding helix; the space is filled primarily by the side chains from the extended N-G linker peptide. Instead, a helix is splayed out, away from the G domain, so that its C terminus is packed against a face of the N-domain helical bundle.

We can propose roles for several of the conserved residues in the G-domain active site by analogy to the structures of other GTPases complexed with bound nucleotide^{16–18}; thus Lys 111 of conserved sequence motif I interacts with the β -phosphate; Arg 138 of motif II stabilizes the γ -phosphate leaving group; Asp 187 of motif III ligates Mg^{2+} , and Arg 191 of motif III stabilizes the nucleophilic water molecule. At the other end of the G domain, Asp 248 of motif IV provides specificity for the guanine base; that this is its function in the G domain of FtsY has been shown by mutation to Asn to yield an

XTP-specific activity⁴. In contrast to these proposed functional roles in interaction with bound Mg^{2+} -GTP, it is striking that, in the structure of the apo form of the NG fragment, the side chains of motifs I, II and III form instead a tight network of interactions among themselves (Figs 2 and 3). Thus the active-site side chains in the apo form of the G domain are effectively sequestered, and a substantial structural rearrangement must accompany the formation of the catalytically competent complex with Mg^{2+} -GTP. Such structural changes may either allow interaction with, or may be made more favourable by interaction with, FtsY or other ligands. In the structures of apo EF-G and magnesium-free EF-G/GDP complex, the corresponding conserved lysine and aspartate residues are in a conformation similar to that seen here¹⁹. Other GTPases are generally unstable in the absence of bound nucleotide. The network of interactions seen here may therefore provide a mechanism for stabilization of the empty state²⁰.

GTPases cycle between an 'activated' GTP-bound state and an 'inactive' GDP-bound state^{9,15}. Relatively small local conformational changes that accompany this 'phosphate switch' are amplified by

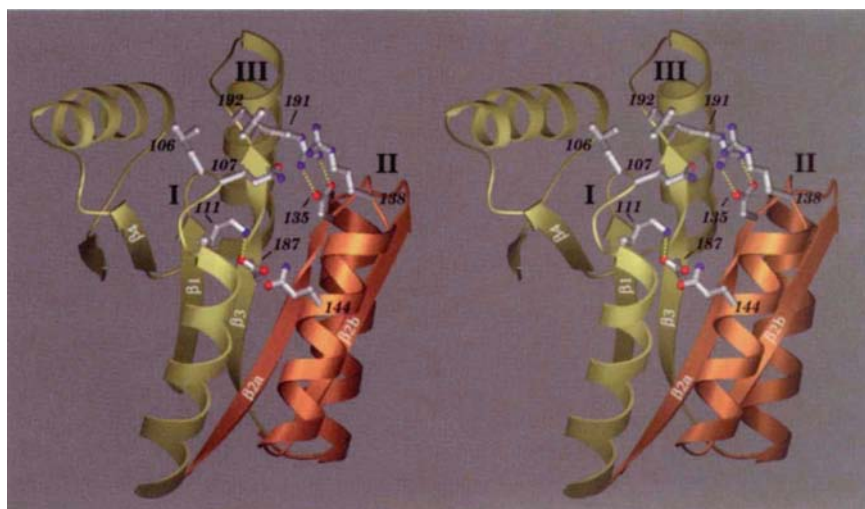


Figure 3 Highly conserved side chains of GTPase motifs I, II and III in the active site of Ffh. In other GTPase structures these side chains interact with the bound ribonucleotide and with the Mg^{2+} required for catalysis, and stabilize the transition state during hydrolysis of the bound GTP^{16–18}. In the apo form of the Ffh NG fragment they form a tight network of interactions. Two in particular are noted: Lys 111, of conserved motif I, forms a salt bridge with Asp 187 of motif III. The Asp in this position has a role in ligating the Mg^{2+} in other GTPase structures¹⁹. Arg 191, also part of motif III, is in the structural position, corresponding to Gln 61 of Ras and Gln

200 of transducin $G\alpha$, which functions to stabilize the attacking water molecule in the transition state^{16,21}. Here it is sequestered by formation of a salt bridge with Asp 135 of conserved motif II. Gln 144 reaches towards the motif I loop and presumably can interact with the α - and β -phosphate oxygens of bound nucleotide. The hydrophobic residues Leu 106 and Leu 192 are exposed to solvent near the active site. Accessible hydrophobic side chains often contribute to protein interaction surfaces (as they do in EF-Tu¹⁷); here they may define part of the region of the NG surface recognized by FtsY or by the C-terminal M domain of Ffh.

Table 1 Crystallographic statistics

Data collection						
Data set	Resolution (Å)	R_{sym} (%)*	Redundancy†	Completeness (%)‡		
Native	2.05	4.6 (13.7)	4.0 (3.9)	99 (98)		
K ₃ IrCl ₆	2.80	4.1 (5.9)	3.2 (3.1)	99 (98)		
Pt(NH ₃) ₂	2.60	5.2 (8.0)	3.3 (3.2)	97 (91)		
PtCl ₄	2.80	5.8 (15.7)	3.6 (3.4)	97 (95)		
K ₂ HgI ₄	2.80	4.7 (6.9)	3.8 (3.8)	97 (96)		
MIR phasing						
Derivative	R_{der} (%)§	Resolution cutoff (Å)	Sites	Phasing power	R_{centric} (%)¶	
K ₃ IrCl ₆	20 (24)	2.8	1	1.82 (1.37)	54 (68)	
Pt(NH ₃) ₂	22 (21)	3.5	4	1.45 (0.83)	61 (80)	
PtCl ₄	15 (22)	3.5	2	1.22 (0.72)	73 (88)	
K ₂ HgI ₄	16 (18)	4.8	4	2.62 (1.34)	42 (78)	
Overall figure of merit to 3.5 Å = 0.64						
Refinement						
	R_{crist} # (%)	R_{free} (%)	Resolution (Å)	Reflections #	Protein atoms	Water atoms
$F > \sigma(F)$	20.2	26.9	20.0–2.05	16,157	2,228	104

Values in parentheses are the high resolution bin.

* $R_{\text{sym}} = \sum |I_n - \langle I_n \rangle| / \sum I_n$, where $\langle I_n \rangle$ is the average intensity over symmetry equivalents.

† Redundancy is the average number of observations of each unique reflection.

‡ Completeness is the fraction of theoretically possible reflections observed at least once.

§ $R_{\text{der}} = \sum |F_{\text{ph}} - F_{\text{p}}| / \sum F_{\text{p}}$, or mean fractional isomorphous difference.

|| Phasing power $\langle \langle f_n \rangle \rangle / \langle E \rangle$, where $\langle f_n \rangle$ is the r.m.s. of the heavy-atom structure-factor amplitude, and $\langle E \rangle$ is the r.m.s. lack of closure error.

¶ $R_{\text{centric}} = \sum |F_{\text{ph}} \pm F_{\text{p}}| - f_n / \sum |F_{\text{ph}} \pm F_{\text{p}}|$ for centric reflections.

$R_{\text{cryst}} = \sum |F_o - F_c| / \sum F_o$. R_{free} was calculated for a subset of reflections (10%) omitted from the refinement.

intra- and intermolecular interactions^{17,21,22}, which centre around the phosphate-binding pocket. The structure of the NG fragment reveals, however, that the N and G domains interact along a distinct face of the G domain distant from the phosphate-binding pocket (Fig. 4). Extensive hydrophobic and electrostatic interactions between the domains are localized to the $\alpha 4$ helix, or 'DARGG' helix, of the G domain and the loop preceding it (Fig. 5), and follow immediately the conserved motif IV (Thr 245 to Asp 248), which mediates interaction with the guanine base. The amino-acid sequence in the interface is highly conserved in all members of the Ffh/FtsY family. In particular, a basic residue corresponding to Arg 252 and an acidic residue corresponding to Asp 42 are almost universally conserved, as are the residues at the positions of Gly 253 and Leu 257. The conservation of this interaction implies a role for this structure in the function of Ffh and its receptor, and suggests that the N domain is responsive to features distinct from the classic 'phosphate switch'. Its close proximity to motif IV, which interacts with the guanine base, suggests that the DARGG interaction is involved in sensing or controlling the nucleotide occupancy of the G domain.

The N and G domains are a structural and functional unit that is conserved between Ffh and its receptor, both proteins functioning along the pathway which targets nascent polypeptides to the membrane. The interaction between Ffh and its receptor, FtsY, both in the GTP-bound form, which causes stimulation of one GTPase by the other, is likely to involve the motif II subdomain, which forms part of the GTPase active site. We can imagine that these regions in Ffh and FtsY, which should exhibit conformational changes upon binding GTP, interact directly. Alternatively, the two proteins might interact in a head-to-tail fashion, with the N domain of one interacting with and stimulating the activity of the G domain of the other. We speculate that, as a consequence of these interactions and the concomitant conformational changes, the affinity of signal sequence binding to the M domain of Ffh is regulated to allow loading and unloading of the nascent chain at defined steps in the targeting cycle. □

Methods

Ffh was cloned from *Thermus aquaticus* and expressed and purified as described (D.M.F. *et al.*, manuscript in preparation). The NG fragment was generated by

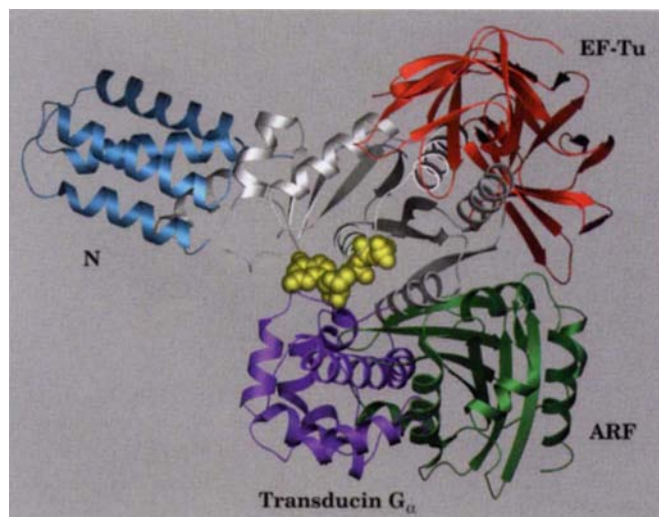


Figure 4 The relative positions, in four different GTPases, of the domains which interact with the GTPase domain of each protein. Shown are the N domain of Ffh; domains II and III of EF-Tu; the dimer interaction of ARF; and the helical insertion domain of transducin G α . Here, the top of the GTPase domain in Fig. 1 has been rotated 90° towards the viewer. The predicted position of bound nucleotide triphosphate is indicated by the yellow space-filling model. The interactions of most known GTPases are localized to the 'phosphate switch' side of the GTPase fold and seem to function by sensing the 'activated', or GTP-bound, state of the protein which causes structural change in that region. In contrast, the N domain interacts at the opposite end of the G domain, suggesting a mechanism that senses or controls nucleotide occupancy distinct from other GTPases.

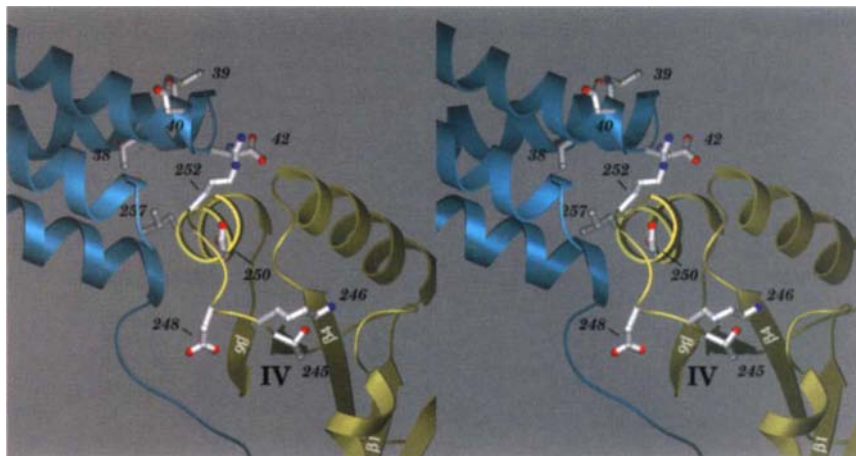


Figure 5 Details of the interface between the N and GTPase domains of Ffh. Highly conserved side chains of GTPase motif IV (residues 245–248), helix $\alpha 4$ (residues 250–257), and the N domain between helices $\alpha N2$ and $\alpha N3$ (residues 38–42) are shown; the C-terminal helix of the G domain is omitted for clarity. In prokaryotes, the sequence of residues 250–254, DARGG, is almost universally conserved. Asp 250 functions to initiate the helix through water-mediated interaction with the helix dipole; Arg 252 of the DARGG motif reaches across between

the side chains of Asp 40 and Asp 42 of the N domain; Gly 253, with $\phi = 104^\circ$, $\psi = 5^\circ$, and Gly 254 allow the close approach of the N and G domains. Leu 257, also highly conserved, inserts into the open end of the N-domain helical bundle and completes that domain's hydrophobic core. The region of the N domain which contributes to this contact is the most highly conserved in the sequence of that domain.

proteolysis, in which protein at 1 mg ml^{-1} in 50 mM Pipes, pH 7.0, 2 mM MgCl_2 , 250 mM NaCl was digested using 0.01 mg ml^{-1} elastase for 1 h, the reaction stopped with 100 mM AEBSE, and the product purified by anion-exchange high-performance liquid chromatography (HPLC). The protein was then concentrated to 30 mg ml^{-1} in water using a Centricon, and crystallization performed in sitting drops over a solution of 30% PEG monomethyl ether 550, 100 mM TAPS, pH 9.0, 200 mM MgCl_2 at room temperature. The crystals grew in space group C2, with $a = 99.9 \text{ \AA}$, $b = 53.9 \text{ \AA}$, $c = 57.4 \text{ \AA}$, $\beta = 119.8^\circ$. As the crystals formed in a cryoprotectant mother liquor, they could be frozen easily, and each dataset was collected from a single crystal in a stream of nitrogen at -170°C . Native and heavy-atom derivative data were collected using MAR30 and RAXIS II image plate detectors mounted on a Rigaku X-ray source. Data were processed using DENZO and SCALEPACK²³ with no sigma cutoff. Subsequent data processing was with the CCP4 package²⁴. Four heavy-atom soak conditions yielded useful derivatives; only one, K_2IrCl_6 , contributed to phasing beyond $3.5 \text{ \AA}$ resolution. Heavy-atom parameters were refined using MLPHARE²⁴. The resolution limit applied to each derivative dataset during phase calculation was determined by inspection of difference Patterson maps and the behaviour of the statistical indicators fh/E and R_{centric} on resolution. The initial MIR electron-density map was calculated at $3.5 \text{ \AA}$ resolution. This map clearly revealed the position of the β -sheet that was expected to form the core of the GTPase fold. An iterative process of building, refinement and phase combination²⁵ yielded a poly-Ala model which accounted for nearly all of the observed main-chain density (although without sequence or correct connectivity). It was refined, in one step, against the full data set and used to calculate a map to $2.05 \text{ \AA}$ resolution. This map enabled us to build the complete amino-acid sequence. The atomic model was built using O²⁶, and the structure was refined using X-PLOR²⁷. Data to a low resolution limit of $20.0 \text{ \AA}$ were included in the refinement after applying a bulk solvent correction. A subset (10%) of the data were set aside for calculation of R_{free} ²⁷ before any crystallographic refinement (including that of the poly-Ala model) was done. The electron-density map is extremely clear, and the polypeptide is generally in well-defined electron density (Fig. 2). The current model extends from Phe 2 to Gly 297; another two or three disordered C-terminal residues may be present. The loop from Gly 271 to Gly 278 cannot be seen in the electron-density map and is presumably disordered. Arg 128 is modelled with two conformations. No Mg^{2+} ions have been located in the structure. The crystals of NG diffract to $1.0 \text{ \AA}$ resolution; a complete analysis of the structure using data to that resolution, including the water structure and the determinants of thermostability, will be presented elsewhere. For comparison with the

structures of other GTPases, the G domains of EF-Tu (left)²⁸, ARF (1hur)²⁹ and transducin $\text{G}\alpha$ (1tnd)¹³ were superimposed on the G domain of Ffh using the motif I loop and β -strands $\beta 1$, $\beta 4$ and $\beta 5$ of the core GTPase fold. The positions of the domains in intra- and intermolecular contact with the GTPase domain are shown in Fig. 4. The structure of EF-G, which was omitted from the figure for clarity, has a G' subdomain that occurs near, but does not overlap with, the position of the N domain in Ffh. Figures were generated using O²⁶ and SETOR³⁰.

Received 27 September; accepted 22 November 1996.

1. Walter, P. & Johnson, A. E. *Annu. Rev. Cell Biol.* **10**, 87–119 (1994).
2. Miller, J. D., Bernstein, H. D. & Walter, P. *Nature* **367**, 657–659 (1994).
3. Kusters, R. *et al. FEBS Lett.* **372**, 253–258 (1995).
4. Powers, T. & Walter, P. *Science* **269**, 1422–1424 (1995).
5. Zopf, D., Bernstein, H. D. & Walter, P. *J. Cell Biol.* **120**, 1113–1121 (1993).
6. Römisch, K., Webb, J., Lingelbach, K., Gausepohl, H. & Dobberstein, B. *J. Cell Biol.* **111**, 1793–1802 (1990).
7. Bernstein, H. D. *et al. Nature* **340**, 482–486 (1989).
8. Römisch, K. *et al. Nature* **340**, 478–482 (1989).
9. Bourne, H. R., Sanders, D. A. & McCormick, F. *Nature* **348**, 125–132 (1990).
10. Pai, E. F. *et al. Nature* **341**, 209–214 (1989).
11. Czworkowski, J., Wang, J., Steitz, T. A. & Moore, P. B. *EMBO J.* **13**, 3661–3668 (1994).
12. Evarsson, A. *et al. EMBO J.* **13**, 3669–3677 (1994).
13. Noel, J. P., Hamm, H. E. & Sigler, P. B. *Nature* **366**, 654–663 (1993).
14. Lütcke, H., High, S., Römisch, K., Ashford, A. J. & Dobberstein, B. *EMBO J.* **11**, 1543–1551 (1992).
15. Bourne, H. R., Sanders, D. A. & McCormick, F. *Nature* **349**, 117–127 (1991).
16. Sondek, J., Lambright, D. G., Noel, J. P., Hamm, H. E. & Sigler, P. B. *Nature* **372**, 276–279 (1994).
17. Berchtold, H. *et al. Nature* **365**, 126–132 (1993).
18. Prive, G. G. *et al. Proc. Natl. Acad. Sci. USA* **89**, 3649–3653 (1992).
19. Al-Karadaghi, S., Evarsson, A., Garber, M., Zheltonosova, J. & Liljas, A. *Structure* **4**, 555–565 (1996).
20. Miller, J. D., Wilhelm, H., Gierasch, L., Gilmore, R. & Walter, P. *Nature* **366**, 351–354 (1993).
21. Milburn, M. V. *et al. Science* **247**, 939–945 (1990).
22. Lambright, D. G. *et al. Nature* **379**, 311–319 (1996).
23. Otwinowski, Z. in *Data Collection and Processing* (eds Sawyer, L., Isaacs, N. W. & Bailey, S.) 55–62 (SERC Daresbury Laboratory, Warrington, 1993).
24. Collaborative Computational Project Number 4. *Acta Crystallogr. D* **50**, 760–763 (1994).
25. Read, R. J. *Acta Crystallogr. A* **42**, 140–149 (1986).
26. Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgaard, M. *Acta Crystallogr. A* **47**, 110–119 (1991).
27. Brünger, A. T. *X-PLOR: A System for X-Ray Crystallography and NMR* (Yale Univ. Press, New Haven, 1992).
28. Kjeldgaard, M., Nissen, P., Thirup, S. & Nyborg, J. *Structure* **1**, 35–50 (1993).
29. Amor, J. C., Harrison, D. H., Kahn, R. A. & Ringe, D. *Nature* **372**, 704–708 (1994).
30. Evans, S. V. *J. Mol. Graph.* **11**, 134–138 (1993).

Acknowledgements: We thank P. Peluso and T. Powers for discussions; P. Focia for assistance with data collection; and H. Bourne for encouragement and comments on the manuscript. This work was supported by fellowships from the Herb Boyer Fund and the Biotechnology Program of the University of California (D.M.F.) and by grants from the NIH (R.M.S. and P.W.).

Correspondence and requests for materials should be addressed to D.M.F. (e-mail: freymann@msg.ucsf.edu). Coordinates and structure factors have been deposited with the Protein Data Bank, accession no. 1ffh.

Crystal structure of the NG domain from the signal-recognition particle receptor FtsY

Guillermo Montoya, Cecilia Svensson, Joen Luijckx* & Irmgard Sinning

European Molecular Biology Laboratory, Structural Biology Programme, Meyerhofstrasse 1, D-69117 Heidelberg, Germany

* Department of Microbiology, Institute of Molecular Biological Sciences, Biocentrum Amsterdam, DeBoelelaan 1087, 1081 HV Amsterdam, The Netherlands

Newly synthesized proteins destined either for secretion or incorporation into membranes are targeted to the membrane translocation machinery by a ubiquitous system consisting of a signal-recognition particle (SRP) and its receptor^{1,2}. Both the SRP receptor and the protein within the SRP that binds the signal sequence contain GTPases^{3,4}. These two proteins, together with the RNA component of the SRP, form a complex^{5–7} and thereby regulate each other's GTPase activity⁸. Here we report the structure of the GTPase-containing portion of FtsY, the functional homologue of the SRP receptor of *Escherichia coli*⁹, at 2.2 Å resolution without bound nucleotide. This so-called NG domain displays similarities to the Ras-related GTPases, as well as features unique to the SRP-type GTPases¹⁰, such as a separate amino-terminal domain, an insertion within the p21^{Ras} (Ras) effector domain¹¹, and a wide-open GTP-binding region. The structure explains the low affinity of FtsY for GTP, and suggests rearrangements that may occur on nucleotide binding. It also identifies regions potentially involved in the transmission of signals between domains and in interactions with regulatory proteins.

The SRP-type GTPases form a distinct subfamily of GTPases¹⁰. The NG domain was identified by sequence alignments^{3,4} in all SRP-type GTPases, and could also be obtained as a stable proteolytic fragment from both FtsY (ref. 7) and SRP54 (ref. 12), the signal sequence-binding protein (Ffh in *E. coli*). In FtsY and its eukaryotic homologue (SR α), the NG domain is linked to a highly charged domain at the N terminus, which in SR α is involved in membrane attachment¹³. SRP54 and its homologues (Ffh in *E. coli*) contain an additional domain at the C terminus (the M domain)^{3,4}, which binds the SRP RNA and signal sequences (for review see refs. 1, 2).

The crystal structure of the NG domain of FtsY (residues 197–497, containing a His-tag at the C terminus¹⁴) was determined by MIRAS (Table 1). It comprises residues 201–495 of FtsY, and 117 water molecules. The structure can be divided into three segments (Fig. 1a): the N domain (197–280), a GTPase domain (291–495), and an α – β – α insertion called the I box, for 'insertion' (333–377). The N domain is a cylindrical module composed of four helices which pack together by interactions between hydrophobic residues that are conserved between members of the SRP family. The interface between the N and G domains is mainly formed by hydrophobic contacts in the interior, and by polar contacts close to the surface. The contact region involves the N terminus, the loop between the helices B and C, plus helices α_6 and α_7 . Helix α_7 fits snugly across helices A and D, and α_6 is wedged between the N terminus and the loop between helices B and C. The N domain is linked to the G domain by an extended arm (residues 280–293) which reaches along the surface of the G domain.

The G domain shows the α – β fold known from H-Ras¹¹ (see Fig. 1b) with the I box in the effector region. The G domain consists of an eight-stranded β -sheet, with seven parallel strands and one

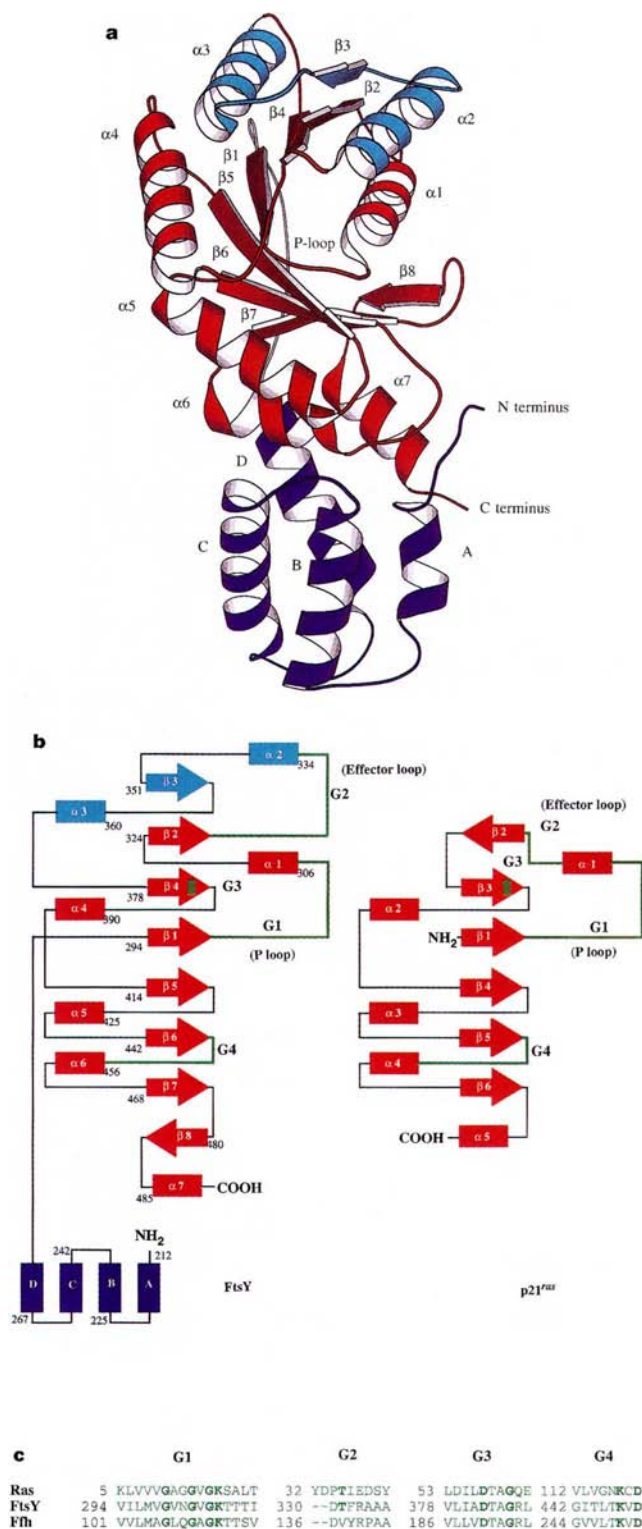


Figure 1 **a**, C α ribbon representation of the NG domain of FtsY drawn with MOLSCRIPT²⁹. The three segments are shown in dark blue (N domain), red (G domain) and light blue (I box). In the N domain, helices are labelled alphabetically in capital letters (A–D). In the G domain, helices and strands are labelled with numbers according to their appearance in the sequence. The P loop and the N and C termini (close in space) are indicated. **b**, Topological diagram of FtsY (left) and p21^{Ras} (Ras)¹¹ (right). Arrows and rectangles represent β -strands and α -helices, respectively. Strands that form a β -sheet are grouped together. The colour code is the same as in **a**. The SRP typical insertion in the effector region is indicated in light blue (I box), and the location of the four consensus elements (G1–G4) is marked in green. **c**, Alignment of the four consensus elements (G1–G4) from Ras¹¹, FtsY and Ffh.

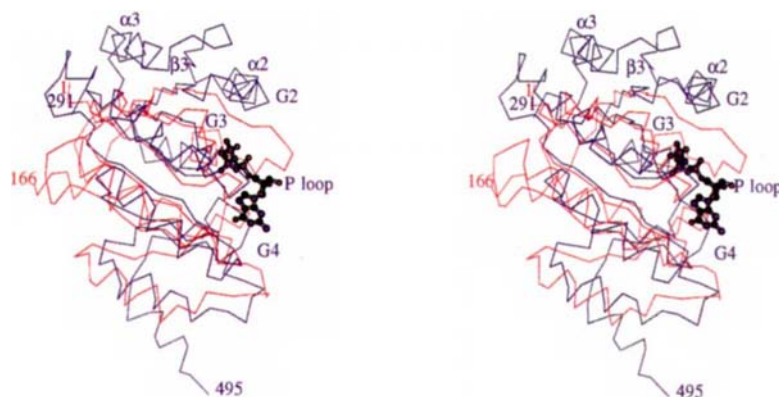


Figure 2 Stereo pair of a C α representation of the G-domain of FtsY (blue) superimposed with Ras¹¹ (red) in complex with GMP-PNP (black). The N and C termini (1 and 166 and 294 and 495, respectively), the four consensus regions and the I box (α 2- β 2- β 3) are indicated. The central β -sheet only shows small

deviations between both structures. The major differences are the I box (at the top) in FtsY and the effector loop in Ras (G2) that closes the binding site. The figure was drawn in MOLSCRIPT²⁹, the superposition was done in O²⁷, and has also been used for Fig. 3. 87 C α atoms are equivalenced with an r.m.s. fit of 1.8 Å.

antiparallel strand, and seven α -helices. The packing of the helices against the sheet is mainly hydrophobic. The regions of best fit, as well as the location of major differences between FtsY and Ras, can be identified in Fig. 2. The most striking difference between FtsY and Ras is the insertion of the I box between the G2 and G3 regions. The I box is a unique feature of the sequence of all SR α and Ffh homologues. The insertion of 29 residues comprises strand β 3, located at the edge of the central sheet, and helices α 2 and α 3, which are relatively exposed. Helix α 3 packs against β 2, β 4 and α 4, whereas α 2 forms only a few contacts with the surrounding protein. Furthermore, α 2 is in a position analogous to the effector loop in Ras-related GTPases. This makes α 2 a good candidate for the site of interaction with a regulatory protein.

The four consensus elements responsible for GTP binding (G1-G4) are conserved in all SRP-GTPases, and show a high degree of

homology to the equivalent regions in Ras¹¹ (see alignment in Fig. 1c). The nucleotide binding site of FtsY is much more open than in Ras, probably because of the absence of bound nucleotide (Fig. 3). Nevertheless, the P loop or G1 region (³⁰⁰GVNGVGKT) adopts an overall structure very similar to that in Ras, in which it is known to interact with the α - and β -phosphate groups of the nucleotide. The P loop in FtsY is stabilized by Asn 302 forming a hydrogen bond to Gly 385, and Lys 306 interacting with Asp 382 and Thr 383.

In the G2 region of FtsY (³³⁰DTFRAA), the peptide chain is orientated in the opposite direction to Ras as a result of the I box. Thr 35 in Ras is known to interact directly with Mg²⁺ and the γ -phosphate of GTP, and to swing away after hydrolysis^{15,16}. The side-chain oxygen of Thr 331 in FtsY is placed about 8 Å further away from the oxygen of the γ -phosphate of GMP-PNP bound to Ras. The side chain of Thr 331 forms hydrogen bonds to the main-chain

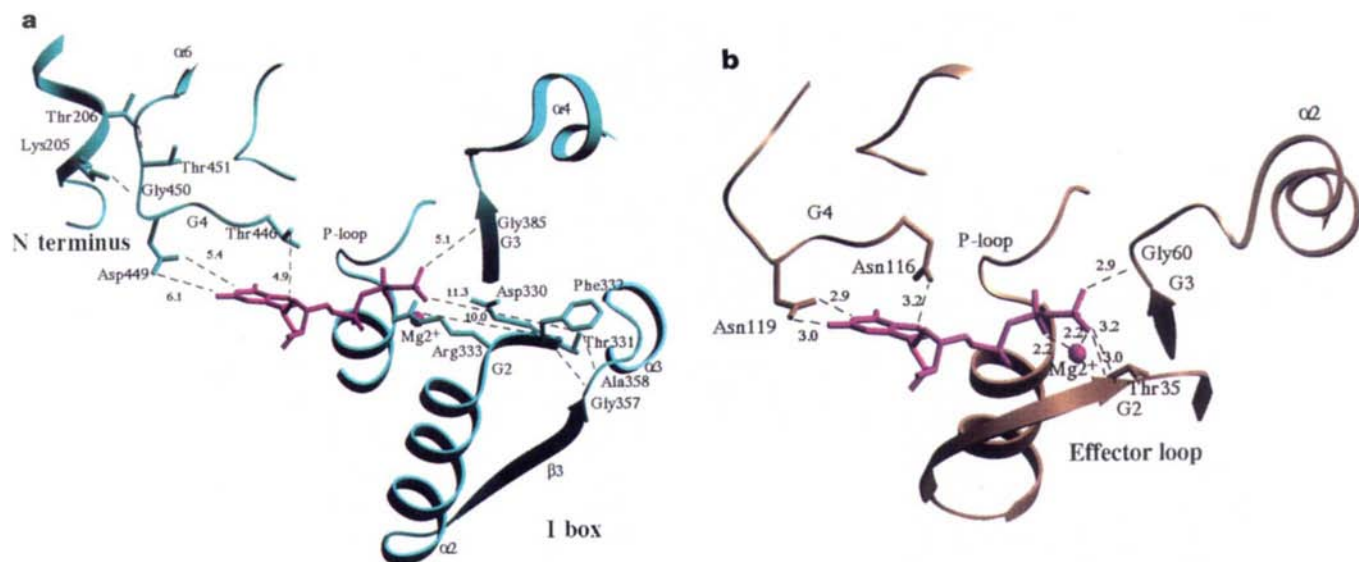


Figure 3 GTP binding site of FtsY (a) and Ras¹¹ (b), shown in the same orientation. The consensus regions (G1-G4) are indicated. Hydrogen bonds between the P loop and the nucleotide have been omitted for clarity. a, GTP binding site of nucleotide-free FtsY (blue). The position of GMP-PNP and Mg²⁺ (purple) was taken from superimposing FtsY on Ras (see Fig. 2). The I box in FtsY is at the lower right corner, the N terminus is at the upper left corner. Distances between GMP-PNP and critical side chains are shown to demonstrate the wide-open binding site compared with Ras. Thr 331 is hydrogen-bonded to Gly 357 and Ala 358. Thr 331 is

hydrogen-bonded to Gly 357 and Ala 358. Asp 330 and Arg 333 are pointing towards the P loop. Asp 449 is about 2.5 Å to the left (compared with Asp 119 in Ras). Stabilizing hydrogen bonds between Lys 205 and Thr 206 with Gly 250 and Thr 251 are indicated. b, GTP binding site of Ras¹¹ (yellow) with GMP-PNP and Mg²⁺ (purple). Residues Asn 119, Asn 116 and Thr 35 are represented with side chains. Hydrogen bonds between the protein and the nucleotide are shown with the distances. This figure was produced using the program SETOR³⁰.

Table 1 Statistics of data collection and structure refinement

Data set	Resolution (Å)	R_{sym} (%)*	Completeness (%)	R_{iso} (%)†	Sites	R_c/R_k ‡	Figure of merit (iso/ano)	Phasing power (iso/ano)§
Native	2.8	3.8	98.0					
Native 1	2.2	6.9	99.6					
(CH ₃) ₃ Pb ₄ Ac	2.8	5.1	98.9	8.7	1	0.51/0.09	0.38/0.34	2.27/2.03
Se-protein	2.8	6.7	98.2	6.3	5	0.13	0.42	2.30
	Resolution range		Reflections/uniques		Protein atoms		Water molecules	R -value/ R_{free} (%)
Refinement	30–2.2 Å		15161/15365		2778		117	22.2/28.0
	Bond lengths		Bond angles		B -factors			
R.m.s. deviation	0.010 Å		1.56°		4.12 Å ²			

^{*} $R_{\text{sym}} = \sum_i \sum_h |I(h) - \langle I(h) \rangle| / \sum_i \sum_h I(h)$, where h is the index for unique reflections, $I(h)$ is the i th measurement of reflection h and $\langle I(h) \rangle$ the mean intensity of all measurements of $I(h)$.
[†] $R_{\text{iso}} = \sum_i |F_{\text{nat}} - F_{\text{deriv}}| / \sum_i |F_{\text{nat}}|$, where F_{nat} and F_{deriv} are the structure factor amplitudes of the native and the derivative, respectively.
[‡] $R_c = \sum_i |F_{\text{deriv}}| / \sum_i |F_{\text{nat}}|$, $R_k = \sum_i |F_{\text{deriv}}| / \sum_i |F_{\text{calc}}|$, where the summations are over the centric reflections, F_{calc} is the calculated structure factor for heavy atoms; $R_k = \sum_i |F_{\text{deriv}}| / \sum_i |F_{\text{calc}}|$, where the summations are over the acentric reflections.
[§] Phasing power = F_p/E , where F_p and E are the r.m.s. of the heavy-atom structure factor and lack of closure error, respectively. $F > 2\sigma(F)$ (all data).

oxygen of Ala 358, while its main-chain oxygen forms hydrogen bonds to the main-chain nitrogen of Gly 357. These interactions with the loop connecting $\beta 3$ with $\alpha 3$ in the I box stabilize Thr 331 in an orientation away from the binding site, which explains the low binding affinity of FtsY for GTP. To bind GTP, Thr 331 has to be released by the I box. Only bacterial Ffh homologues replace Thr by a hydrophobic residue in the G2 regions, which is always accompanied by the replacement of the following Phe 332 by Tyr (Fig. 1c). The role played by Thr 331 in nucleotide binding might be taken over by Tyr. The side chain of Phe 332 in our structure points out to the surface of the protein and interacts with residue 237 in the interdomain interface of a neighbouring protein molecule in the crystal lattice. This might be an artefact of crystal packing, although it might also have functional significance.

The G3 region of FtsY (³⁸²DTAGR³⁸⁶) superimposes well with that of Ras. Residues 385–395 are less well defined than the rest of the structure, with high temperature factors (52 Å² compared to 24 Å² overall). Similarly, the highest temperature factors and multiple conformations are found in the corresponding region, 60–70, in Ras. This region (the so-called switch II region) in Ras dramatically differs between GTP- and GDP-bound forms^{17,18}.

The characteristic sequence of the G4 region is a set of four hydrophobic residues followed by the motif NK × D in all but SRP-GTPases, in which Asn is replaced by Thr. Asp 119 in Ras binds to the guanine ring and is responsible for nucleotide specificity¹⁹. The G4 region in FtsY (⁴⁴⁶TKLD) has the same overall orientation as in Ras. Changing Thr 446 into Asn decreases GTP binding⁷, whereas substitution of Asp 449 with Asn changes the nucleotide specificity from GTP to xanthosine-triphosphate (XTP)⁸. This suggests that the G4 region in FtsY also binds the guanine ring of the nucleotide. However, Asp 449 is displaced by about 2.5 Å (Cα–Cα distance) from the corresponding Asp 119 in Ras (Fig. 3). To interact with the nucleotide, Asp 449 has to move into the binding site. This movement would affect the N terminus of the N domain, as it is hydrogen-bonded to the main chain of residues Gly 450 and Thr 451 by means of the side chains of residues Lys 205 and Thr 206, respectively. In support of this, the N domain is susceptible to protease digestion in the absence of nucleotide, but is protected when GTP or GDP are bound⁷.

Based on the high sequence identity between the NG domains of SRP-GTPases (about 34% between FtsY and Ffh), and similar secondary structure predictions, the structure of the NG domain of Ffh and SRP54 can be modelled on our structure. Because the N and C termini of the NG domain are close together, it is tempting to assume that the N-terminal acidic domain in FtsY (SRα) and the C-terminal M domain in Ffh (SRP54) occupy similar positions relative to the NG domain. The role of the N domain could be to orientate these domains relative to each other or to transfer signals between them. The latter possibility is favoured because not only do FtsY and

Ffh/4.5S RNA have to be released from one another after GTP hydrolysis, but the ribosome-nascent chain complex also has to be released from Ffh. GTP-FtsY has a conformation that is competent for binding Ffh/4.5S RNA, which activates GTP hydrolysis by Ffh⁸. It is not yet known how FtsY and Ffh interact, or which domains are involved in complex formation. In the structure of a Ras-type GTPase in complex with its effector (Rap1A with the Ras-binding domain (RBD) of c-Raf1) the interface is formed by the last antiparallel strand of the β -sheet of Rap1A and a β -strand of the effector²⁰. A similar mode of binding might be used by FtsY and Ffh. The location of the I box in FtsY in the region equivalent to the effector loop in Ras makes it a good candidate for the area of interaction with Ffh. The I box contributes one strand to the sheet in FtsY, which is reminiscent of the Rap1A–RBD interaction. Therefore the I box could also be described as a built-in factor that stabilizes the nucleotide-free form of FtsY. Assuming that FtsY and Ffh have evolved from a common ancestor, the I box must have been acquired before diverging and further modifications were necessary to prevent them from stimulating their own GTPase. To bind GTP, a conformational change is needed which might be induced by the interaction of the I box with a regulatory protein, such as Ffh/4.5S RNA. In the case of SRP54, the ribosome acts as effector, as it stimulates GTP binding to SRP54 (ref. 21). The ribosome may also promote GTP binding to the other SRP-GTPases, including FtsY. Our structure will assist in designing mutants and hybrid proteins for testing the proposals made above, and for elucidating the mechanism by which FtsY and Ffh/SRP-RNA (and their homologues) form heterodimeric complexes in the presence of GTP^{5,6} and stimulate each other's GTPase activities⁸. □

Methods

Preparation of derivatives and data collection. Crystals of the NG domain of FtsY were obtained as described¹⁴. The crystals belong to the monoclinic space group $P2_1$ with cell dimensions $a = 32.2$ Å, $b = 79.57$ Å, $c = 59.21$ Å and $\beta = 94.45^\circ$, and contain one molecule per asymmetric unit with a Matthews coefficient of 2.3 Å³ per Da. One useful derivative was obtained by soaking of the native crystals in mother liquor containing 10 mM (CH₃)₃Pb₄Ac for 4 days, which gave a unique site. Seleno-methionine protein was prepared as described¹⁴. Native and the Pb derivative data were collected in-house using a rotating anode. Native data set 1 was collected on the BL4 beamline at the ESRF with a wavelength of 0.99 Å. Anomalous data were collected for all derivatives. Data were processed using the programs DENZO and Scalepack²².

Patterson solution and phase calculation. Isomorphous difference Patterson maps at 4-Å resolution for the Pb derivative provided a clear solution for one site. Anomalous difference Patterson for this derivative yielded a solution consistent with the isomorphous difference Patterson function. Cross-difference Fourier maps using the phases of the Pb derivative revealed the positions of five Se atoms in the Se derivative. The side chain of the sixth methionine was not defined well enough to reveal the position of the last Se

atom. Heavy-atom parameters were refined using PHASIT in PHASES package²³. Initial MIRAS phases at 2.8 Å resolution using both isomorphous and anomalous data were obtained. Solvent flattening procedures as implemented in PHASES²⁴ were used to improve the map. A molecular mask was built using a solvent content of 37% in the unit cell. The resultant electron-density map with a figure of merit of 0.88 allowed us to place 280 Cα carbons of 307 residues. Only one loop had disconnected density (region 385–395 in the protein sequence). The model contains 295 of 307 residues. Therefore, only four residues at the N terminus and eight at the C terminus (six of which are histidines) are disordered. The first 10 residues (201–211) are not well defined, perhaps because of the missing stabilizing interactions with the acidic domain and/or because no nucleotide is bound.

Refinement. The model was refined using the slow-cooling protocol in XPLOR²⁵ and rebuilt against $2F_o - F_c$ maps using R_{free} as a monitor of model quality, followed by positional refinement with bulk solvent correction. Solvent water molecules were identified using the program ARP²⁶ and checked in the map using the WAT_PEPKIP routine in O²⁷ with cross-validation R_{free} . The overall B-values were refined isotropically with an average B of 24.72 Å². The Ramachandran plot (not shown) showed that over 91% of the main-chain torsion angles are in the most favoured regions, with no amino acids in disallowed regions. The geometric parameters were checked with the program WHAT_CHECK in the WHAT_IF package²⁸.

Received 19 September; accepted 21 November 1996.

1. Walter, P. & Johnson, A. E. *Annu. Rev. Cell Biol.* **10**, 87–119 (1994).
2. Lütcke, H. *Eur. J. Biochem.* **228**, 531–550 (1995).
3. Römisch, K. *et al. Nature* **340**, 478–482 (1989).

4. Bernstein, H. D. *et al. Nature* **340**, 482–486 (1989).
5. Connolly, T., Rapijko, P. J. & Gilmore, R. *Science* **252**, 1171–1173 (1991).
6. Miller, J. D., Bernstein, H. D. & Walter, P. *Nature* **367**, 657–659 (1994).
7. Kusters, R. *et al. FEBS Lett.* **372**, 253–258 (1995).
8. Powers, T. & Walter, P. *Science* **269**, 1422–1424 (1995).
9. Lührink, J. *et al. EMBO J.* **13**, 2289–2296 (1994).
10. Bourne, H. R., Sanders, D. A. & McCormick, F. *Nature* **349**, 117–127 (1991).
11. Pai, E. F. *et al. EMBO J.* **9**, 2351–2359 (1990).
12. Römisch, K., Webb, J., Lingelbach, K., Gausepohl, H. & Dobberstein, B. *J. Cell Biol.* **111**, 1793–1802 (1990).
13. Young, J. C. *et al. J. Biol. Chem.* **270**, 15650–15657 (1995).
14. Montoya, G., Svensson, C., Lührink, J. & Sinning, I. *Proteins Struct. Funct. Genet.* (in the press).
15. Schlichting, I. *et al. Nature* **345**, 309–315 (1990).
16. Milburn, M. V. *et al. Science* **247**, 939–945 (1990).
17. Tong, L. A., deVos, A. M., Milburn, M. V. & Kim, S.-H. *J. Mol. Biol.* **217**, 503–516 (1991).
18. Wittinghofer, A. & Pai, E. F. *Trends Biochem. Sci.* **16**, 382–387 (1991).
19. Schmidt, G. *et al. Oncogene* **12**, 87–96 (1996).
20. Nassar, N. *et al. Nature* **375**, 554–560 (1995).
21. Bacher, G., Luetcke, H., Jungnickel, B., Rapoport, T. A. & Dobberstein, B. *Nature* **381**, 248–251 (1996).
22. Otwinowski, Z. in *Proceedings of the CCP4 Study Weekend* (eds Sawyer, L., Isaacs, N. W. & Bailey, S.) 55–62 (SERC Daresbury Laboratory, Warrington, UK, 1993).
23. Furey, W. *Am. Crystallogr. Assoc. 40th Aniv. Meeting* (New Orleans, LA, 1990).
24. Wang, B. C. *Methods Enzymol.* **115**, 90–115 (1985).
25. Brünger, T. A. *XPLOR Version 3.1* (Yale University, 1993).
26. Lamzin, V. S. & Wilson, K. S. *Acta Crystallogr. D* **49**, 129–147 (1993).
27. Jones, T. A. *et al. Acta Crystallogr. A* **47**, 110–119 (1991).
28. Vriend, G. *J. Mol. Graph.* **8**, 52–56 (1990).
29. Kraulis, P. J. *J. Appl. Crystallogr.* **24**, 946–950 (1991).
30. Evans, S. V. *J. Mol. Graph.* **11**, 134–138 (1993).

Acknowledgements: We thank A. Thompson and B. Rassmussen from the EMBL outstation in Grenoble for help with the data collection; the ESRF for access to the MAD-beamline, BM-14 and BL-4; H. Lütcke for discussions and reading the manuscript; and H. Savage for suggestions during refinement.

Correspondence and requests for materials should be addressed to I.S. (e-mail: sinning@embl-heidelberg.de). The coordinates have been deposited in the Protein Data Bank (1fts)

KNOW YOUR COPY RIGHTS RESPECT OURS

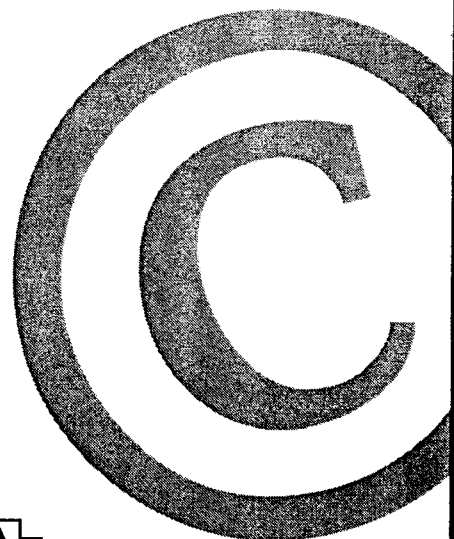
The publication you are reading is protected by copyright law. This means that the publisher could take you and your employer to court and claim heavy legal damages if you make unauthorised photocopies from these pages.

Photocopying copyright material without permission is no different from stealing a magazine from a newsagent, only it doesn't seem like theft.

The Copyright Licensing Agency (CLA) is an organisation which issues licences to bring photocopying within the law. It has designed licensing services to cover all kinds of special needs in business, education, and government.

If you take photocopies from books, magazines and periodicals at work your employer should be licensed with CLA.

Make sure you are protected by a photocopying licence.



The Copyright Licensing Agency Limited
90 Tottenham Court Road, London W1P 0LP

Telephone: 0171 436 5931
Fax: 0171 436 3986

A new look for the market

This product update shows its new style but maintains its old function of providing information on products, such as a video tape analysis system, new autofluorescent proteins, and a microplate and reagent handling system.



B 15 incubator optimizes space with new racks.

B 15 compact incubator

From Heraeus Instruments

Stacking kits to ensure full exploitation of compact incubator capacity

Heraeus has introduced insertable racks for its B 15 compact incubator, allowing more economical use of the interior space. The new stacking kit expands the B 15's capacity to 72 nutrient medium carriers, all of which may be incubated simultaneously. These autoclavable stainless steel racks can be plugged into each other and transported in stacks, each rack handling six samples. The incubator has room to accommodate twelve of these racks, three of them side by side and four on top of each other. The incubator has fifteen litres of internal volume and can utilize sample tubes of various sizes. The B 15 can also be used in tempering, warm storage and drying at temperatures of up to 50 °C.

Reader Enquiry No. 100

The Observer

From Noldus Information Technology.

A new video tape analysis system for observational analysis and documentation

This video tape analysis system operates in a Windows environment and has been designed for collecting, editing, and analysing observational data from video tape. The new features lead to an increased ease of use, significant time savings and improved management of observational data. The video image appears in a window on the computer screen using a video overlay board, as an alternative to a video monitor. This prevents 'look-away' errors and reduces data-entry errors, as well as overall system cost and space requirements.

In addition, overlay boards allow the user to grab video images and store them in disk files for future use as illustrations. To locate material the user can search for a video time (for example, 02.24:36.04), a marker (or any previously entered free-format annotation), a scored event (for example, a person, activity) or text. The user simply specifies the item for which to search, the program then displays a cursor line next to the target record. The user can then click a button 'show video' to spool the tape to the corresponding video image. An edit decision list (EDL) is used as a script that determines which episodes on one or more source appear on the destination tape and in what order. It can be saved in different formats, for direct import into a video editing system.

Reader Enquiry No. 101

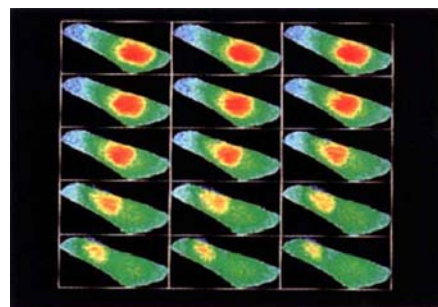
Imaging items

Merlin low-light imaging system

From Olympus Precision Instrument Div.

A high-speed, real-time, low-light imaging system for the measurement of intracellular ions in living cells and tissues

With Merlin, full ratiometric fluorescence imaging capability is provided for all currently available fluorescence probes, including Fura2, Indo 1, BCECF, Calcium Green, FuraRed and the SNARF and SNAFL dyes. It is also said to process up to four ratio pairs in real-time, allowing the user to keep pace with rapid advances in probe technology. The imaging system is one of a range of microscope-based integrated imaging and photometric systems for cellular physiology, resulting from an alliance between Olympus Precision Instrument Division and Life Science Resources. The package consists of a high-speed, low-light level imaging camera (analog or digital), an ultra-fast monochromator, a high-end Windows-based PC workstation with mass optical storage and all optical components for interfacing with the microscopy system. The range of analog and digital cameras provides a choice of speeds between 10 and 100 frames per second and resolution up to 1,024 × 1,024 pixels. Cooled, intensified analog cameras are available with integrating capabilities, providing low-noise images with an improved dynamic range. Digital cameras are also available offering high dynamic range from 8–14 bits per pixel. Continuous wavelength ranges from 300 nm to 800 nm are possible with the SpectraMaster monochromatic light source, which allows wavelength shifts of 100 rapid wavelength



Merlin full ratiometric imaging system.

changes, achieved in as little as 5 ms. These wavelength changes are achieved with high-speed, galvanometer-control technology.

Reader Enquiry No. 102

Autofluorescent proteins

From Quantum Biotechnologies

Blue fluorescent protein and the bright green or red-shifted mutant fluorescent proteins

The company has added four new AFPs vectors to the molecular biology tool box. The pQBI63 and pQBI67 carrying the coding sequences for green fluorescent protein (GFP) and blue fluorescent protein (BFP), respectively, are under the control of T7 promoter, allowing overexpression of the proteins in *Escherichia coli*. The pQBIK25 holding the GFP coding sequence and a 'neofusion' sequence, under the control of the mouse PGK promoter, represents a useful tool for selection. Additionally, the pQBI-PolII vector holds the GFP-neofusion and is under the control of PolII promoter, offering another solution for stable expression and stable cell lines.

Reader Enquiry No. 103

Gridded cover slips

From Electron Microscopy Sciences

Gridded microscope slides and coverslips for efficient location of specimens

The coverslips are 2.5 cm × 2.5 cm squares, 0.5-mm in thickness and imprinted with a 5-mm diameter centre ring. In the centre of the ring, on the reverse side of the coverslip, there is a 1 mm × 1 mm laser-etched ruled grid. The grids are divided into 100 small squares, each measuring 0.1 mm × 0.1 mm. Cells can be studied and areas of interest located much easier and faster. The microscope slides, which are available in four different types are 3 cm × 2.5 cm and have an indexed grid on the reverse side of the frosted end. The ink is resistant to solvents and stains. Moreover, using these slides should make it

easier to relocate individual organisms or cells. There are 81 squares in total, which are available uncoated, coated with poly-L-lysine or silane, and with round or square wells.

Reader Enquiry No. 104

Bio-Rad MRC1000

From Bio-Rad

GFP in confocal microscopy study of viral invasion and spread through plant tissues

A new application note describes the use of confocal microscopy and green fluorescent protein (GFP) for research into the progress of plant viral invasion at a cellular level. It describes how researchers at the Scottish Crop Research Institute used GFP as a non-invasive, *in vivo* marker of gene expression to study virus infection in tobacco plants. In this study, plants were infected with a potato virus X vector containing the GFP marker. The spread of viral infection was monitored by microscopic examination of leaf cells using a Bio-Rad MRC1000 confocal imaging system. The use of confocal microscopy allows large fields of view to be imaged under low magnification, removing out-of-focus flare and digitized imaging to produce clear images. The method provides not only a useful means of tracing viral invasion, but also enables the study of plants induced to synthesize exogenous proteins.

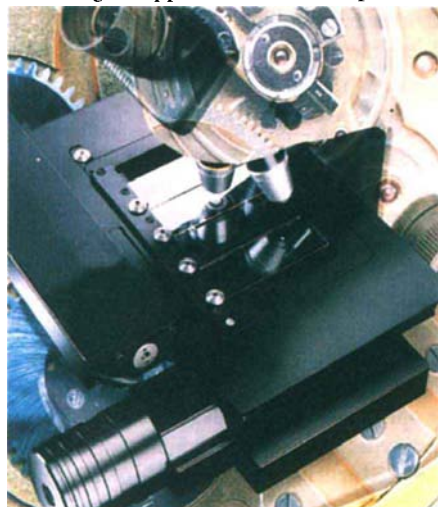
Reader Enquiry No. 105

Plant root systems observer

From Prior Scientific

Improve productivity in microscopy and image analysis applications using a ProScan motorized microscope stage

This stage is designed to improve productivity, to allow automated analysis and to minimize the risk of repetitive strain injuries. ProScan is manufactured with high quality mechanics, resulting in smooth and precise stage movements. A variety of stages is available for most microscope systems and for a wide range of applications. ProScan provides



Prior Scientific's mechanized microscope stage.

fully programmable sample positioning and the ability to return quickly and precisely to areas of interest. The system is compatible with a variety of image analysis software programs and a comprehensive software command set is available for OEM applications.

Reader Enquiry No. 106

ProScan motorized stage

From University of Leeds Innovations

Help the observation of root systems development in vivo

The problem for researchers and growers has been that Petri dishes and magenta pots do not facilitate the observation of root systems *in vivo*. This group has produced a simple solution, which allows easier examination of any part of a plant root system and its interaction with the environment, at either the macro- or microscopic level. Easy observation of root systems is made possible by containing a chosen growth medium between two transparent plates and effectively growing the plant root system 'two dimensionally'. The compatibility of this growth unit with microscopic examination facilitates the use of all microscopic conditions, including fluorescence. The ability to visualize an entire root system, or any part of it, under fluorescent conditions makes this unit suitable for the study of continual reporter gene (GFP) expression. The unit can also be used with real-time video. The aerial part of the plant is maintained in a 'trough', integral to the unit, to allow normal growth and development. As it can be sterilized, the unit is suitable for tissue culture and any growth medium can be incorporated. The unit can also be used for observing effects in soil-grown plants.

Reader Enquiry No. 107

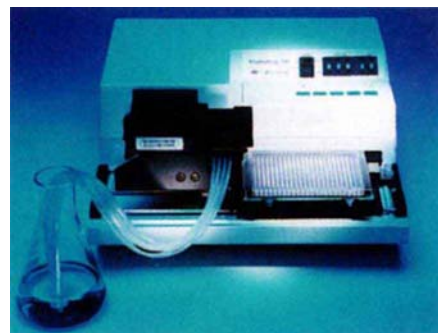
Liquid handling

Multidrop-384 reagent dispenser

From Labsystems and Denley

A 384-well reagent dispenser for the miniaturization of assays

The use of 384-well plates should enable laboratories to use smaller samples, save on reagent costs and increase throughput, particularly in screening applications. The Multidrop-384 reagent dispenser is designed to eliminate the need for labour-intensive manual pipetting. It is able to switch from the traditional 96-well to 384-well plates and can dispense up to eight different liquids through its eight-channel manifold. Any number of columns can be selected, from 1–24 for 384-well plates and from 1–12 for 96-well plates. Dispensing volumes range between 5 µl and 395 µl for 96 wells and 5 µl and 100 µl for 384 wells. It offers a stated accuracy of ± 2 per cent across all volumes, as well as fast operation, filling 384 wells in less than a stated 20 seconds and 96 wells in 5 seconds. It can operate either as a standalone instrument using a simple



Labsystems' Multidrop-384 for reagent handling.

keypad and clear LED display or through a PC, allowing time-controlled dispensing.

Reader Enquiry No. 108

LISSY

From Zinsser Analytic

A robotic sample processing pipetting system for automated high-throughput sample processing applications

This system is designed to save labour, increase precision and help to improve the quality of the results. Lissy is supplied with basic software that can readily automate almost any laboratory pipetting protocol, and can be enhanced by specific application protocols. It uses four sampling tips to pipette in several formats, for example, tube to tube or tube to microplate, and can perform up to 1,500 pipetting steps per hour. In addition to sample pipetting, it can add reagents, perform predilution and serial dilution, as well as automate other functions. Cross contamination and carryover are said to be minimized to below detectable limits through the optimization of the individual liquid-level sensors in the tips, and by a pulsating tip wash (washing the tip inside and outside). Contamination and carryover can be eliminated by utilizing the disposable tip option. Volumes from 5 µl to many millilitres can be pipetted precisely by the action of four high-precision syringe pumps. Any sample and reagent vessel can be used on the open work surface, and standard racks accommodating tubes, bottles and microplates are also available.

Reader Enquiry No. 109

fmax fluorescence microplate system

From Molecular Devices

A microplate reader that combines software and fluorescence microplate measurement

The fmax is a 96-well format fluorescence microplate system that is stated to make readings in only 25 s, making it suitable for kinetic applications, as well as more common endpoint methods. The semiconfocal optical design can be configured for either top-down or bottom-up reading in a matter of minutes. In the bottom-up configuration, the reader is suited for applications where the fluorescent probe is bound directly to cell

monolayers. An optional incubator is designed to ensure that cell cultures are at the required temperature while in the reader. The system includes Softmax Pro software for microplate analysis, providing not only calculation capabilities but also graphics and text processing. There is said to be no need for data export, as the user can generate complete and detailed data spreadsheets, graphic representations and a written report. This software is available for both the Macintosh and Windows95 operating systems.

Reader Enquiry No. 110



fmax for kinetic and end-point applications.

Instrumentation

DuraSamplIR

From ASI

A sampling accessory designed for reaction monitoring with FT-IR spectrometers

This technology is for use with all FT-IR spectrometers and is now available with DuraDisk interchangeable sampling plates. The DuraSamplIR is an ATR-based device that uses the SensIR diamond-composite technology to provide improved sampling versatility and reproducibility. DuraDisk permits users to customize the DuraSamplIR to best address specific sampling requirements across a broad range of physical and chemical compositions. The ATR element in all sampling plates is diamond, providing an abrasion- and chemical-resistant sampling surface. This should allow an FT-IR to analyse a wider array of samples, including abrasive powders, hard materials and corrosive liquids, as well as films, pastes, gels and all aqueous/non-aqueous liquids. The sampling plates permit a user to vary the optical properties of the ATR crystal for optimum measurement performance with individual sample types. The interchangeable plates supply a true 6,000 to 400 wavenumber measurement range, and even make possible the analysis of carbon black filled polymers. The small sampling spot on each DuraDisk assures consistent sample contact with the crystal, providing enhanced measurement accuracy and repeatability. An optional temperature control unit provides accurate sample heating up to 100 °C.

Reader Enquiry No. 111

UV Solutions

Hitachi Instruments

Instrument control software for the company's ultraviolet/visible spectrophotometers

This software is based on the Microsoft Windows95 or Windows NT platform, offering 32-bit processing and a true multitasking capability. This allows the user to process data while running more than one instrument, to control several systems from the same computer at the same time or to run a variety of software packages to generate custom reports. The post-run processing, with in-place object linking and embedding (OLE), enhances the spectral presentation and provides a wide range of arithmetic and data-management functions. The model U-2010 features double-beam stability, a large sample compartment and a compact footprint. The Model U-3010 offers features like low stray light, variable bandpass and high energy instrumentation, with linearity greater than 3.0 absorbance. The standard sample compartment accommodates a wide range of accessories to address automated analyses, specular and diffuse reflectance, turbid samples, thin films, long path cells, and continuous flow-through monitoring.

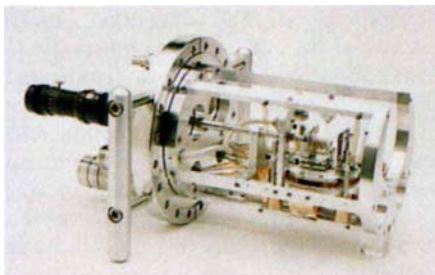
Reader Enquiry No. 112

AutoProbe VP2

From Park Scientific Instruments

A scanning probe microscope for atomic-scale surface analysis in ultra-high vacuum

The instrument combines high-temperature imaging with three-dimensional probe positioning, advanced spectroscopy functions and self-sensing AFM probes. It is available in three system configurations: standard, extended y-translation and high temperature. This ultra-high vacuum (UHV) SPM offers high-temperature operation in both scanning tunnelling microscopy (STM) and atomic force microscopy (AFM). According to the company, this instrument can produce AFM images up to 300 °C and STM images up to 600 °C. The microscope provides both direct and indirect heating capabilities up to 1,200 °C for sample annealing. Variable temperature studies in AFM and STM will yield new information on phenomena, such as phase transitions between surface melting and crystallization, step formation, defect



Ultra-high vacuum SPM and STM analysis.

growth and surface recrystallization. Direct applications include high-temperature analysis of the fundamental physics of surface absorbates, novel semiconductor compounds and atomistic studies of chemical interaction. The open architecture of the microscope head permits sample transfer from several directions, as well as *in situ* interchange of AFM and STM probes. It also incorporates piezolevers, self-sensing AFM cantilevers that should eliminate the complicated alignment procedures required by laser beam bounce detection systems.

Reader Enquiry No. 113

Spectromass 2000 ICP-MS

From Spectro Analytical Instruments

Automated multiple element analysis by inductively coupled mass spectroscopy

This ICP-MS instrument should help provide flexible performance for the automated analysis of multiple elements at picogram per litre levels. According to the manufacturer, the instrument's stability in regard to matrix effects makes it a universally applicable measuring instrument — one with an extremely high detection capacity that can be achieved even under 'real' laboratory conditions. The detection capacity and improved linear measuring range is achieved by coupling the ion source of the ICP to a fast quadrupole mass filter, as detector, through a specially constructed vacuum interface. The RF generator and the HMC cones are said to allow for work with high-saline solutions without compromising instrument stability and with no danger of sampler blockage. Windows-based, multitasking SmartAnalyser software is said to be user friendly and highly flexible.

Reader Enquiry No. 114

Mercury NMR spectrometer

From Varian

Designed to be an affordable, easy-to-use nuclear magnetic resonance spectrometer

Mercury combines small size (stated to be 50 per cent smaller) and high resolution in a superconducting NMR spectrometer for a wide range of industrial and academic applications. According to the manufacturer, this is the first NMR spectrometer to be offered in this price range, which includes a four-frequency console as a standard feature. The system is computer interchangeable and provides automated spectra of four nuclei without further tuning. In addition, there is an optional broadband configuration that allows the analysis of almost any active NMR nucleus. The system is said to offer high-quality spinlock analysis (TOCSY and ROESY), coupled with the pulsed-field gradient Performa I system (optional). It is designed for universities or high-throughput industrial environments with a user friendly interface to simplify operation for novices.



Varian's Mercury NMR with Glide software.

The Glide software uses a graphical user interface to walk users through experiments step by step. Routine two-dimensional NMR experiments, such as heteronuclear correlation and COSY, are preprogrammed and fully automated. For advanced users, Glide can be customized to individual requirements and accommodates a wide variety of sample and solvent types.

Reader Enquiry No. 115

Argus-50/2D luminometer

From Hamamatsu

Luminometer for the detection and measurement of chemiluminescent signals

Designed to provide rapid detection and measurement of chemiluminescent signals, this system is based on an ultra-high-sensitivity video camera that can detect individual photons from a blotting sheet or microplate. Photons are then integrated into a frame memory to produce a luminescent image. A dedicated software package is offered, allowing efficient quantitative analysis of band patterns and comparisons between lanes. The software for this package allows for simultaneous microplate measurement up to 96 wells. This includes enzyme immunoassay function and the analysis of kinetic changes for applications such as phagocytosis measurement of white blood cells. This series can be configured for FISH, intracellular Ca^{2+} measurement and gene expression using luciferase.

Reader Enquiry No. 116

Software

WinLIMS

From QSI

An open architecture laboratory information management system for Windows

This system provides a choice of SQL database engine, operating system, hardware platform and graphical user interface. The system was designed as a 'client server' with 'open architecture' in a Windows-based user interface, providing central storage for all analytical data (including imports from instruments and other software packages). It maintains specifications, methodologies and instrument records in a controlled manner, as dictated by ISO 9000. Reports can be configured by the user or can be preconfigured. Certificates of analysis (COA), worklists and the like

are said to be produced efficiently with desktop publishing quality. Integration for sharing data with popular PC applications is executed using DDE, OLE, 'cut and paste', SQL and export functions. WinLIMS can also be integrated with mission critical applications, such as MRP, using SQL tools. The program uses Windows95, 3.11, NT or OS/2 Presentation Manager as its interface, as well as incorporating fourth generation tools, thereby reducing the cost of the configured LIMS.

Reader Enquiry No. 117

Worklist Manager for LabManager

From Beckman Instruments

An integral option for the company's C/S laboratory information system (LIMS)

The worklist concept is based on the grouping together of logically related samples that are to be analysed in the laboratory. The system includes a graphical user interface that provides convenient access to functions, commands and worklists. Worklist and candidate sample pages are tabbed so that multiple worklists can be open at one time and are readily accessible to the user. Other features include custom presentation and retrieval formats, automatic assignment of samples for standards and controls, and automatic addition of tests for duplicates and/or spiked samples. The program also has three levels of access so that the routine operator, the laboratory supervisor and the system administrator have different levels of control over the preparation or editing of a worklist.

Reader Enquiry No. 118

D-Fence 4

From Sophos

D-Fence disk authorization software, includes high security disk encryption

This software is designed to protect data stored on PCs and laptops in the event of the machine being lost, stolen or sent for repair. According to the manufacturer, the program protects data on floppy disks and hard disks using a 'strong' encryption algorithm. In addition to disk encryption, it offers two options for controlling disk traffic entering or leaving an organization: only authorized, encrypted floppies can be used. D-Fence 4 also allows the erased area of floppy disks to be purged to prevent data being smuggled in and out of organizations via erased files. It is said to be simple to install and to use, and has low memory and processing requirements. Separate versions of D-Fence 4 are available for commercial and UK government use. The government version uses a UK government-specific algorithm, whereas the commercial version uses the highly secure 674-bit SPA block cypher.

Reader Enquiry No. 119

Chemometrics training

From Infometrix

A three-day course emphasizing hands-on training with practical chemometrics

Infometrix has expanded its services in chemometrics training with a course that covers data collection for multivariate analysis, model optimization and model building, classification, and regression and data exploration techniques. The training provides students with experience using the company's Pirouette chemometrics software (participants are encouraged to bring their own data for individual instruction). Courses are provided at Infometrix' home office in the Seattle, Washington area or they can be provided at the customer's location. Scheduled dates for the Seattle courses are April 16-18, June 4-6 and October 8-10, 1997. Custom and private data set analysis and consultation will also be available during the course.

Reader Enquiry No. 120

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

ADVERTISEMENTS

PNA
TIB MOLBIOL

Order
per fax...
+49 30 78 79 94-99
or e-mail
DNA@TIB-MOLBIOL.DE

READER ENQUIRY NO. 86

Check out
our homepage at
<http://www.resgen.com>

Peptides
\$18/residue

Antibodies
monoclonal - \$8500
polyclonal - \$1195

Complete services for your
custom peptide and polyclonal or
monoclonal antibody needs.

Research Genetics, Inc.
U.S. or Canada 800-533-4363
U.K. 0-800-89-1393
FAX 205-536-9016

READER ENQUIRY NO. 87